

INFLUENCE OF ENTRAPMENT AND SURFACE ADSORPTION
ON THE CONVERSION CAPACITY OF PSEUDOMONAS SPP

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Department of Applied Microbiology
Chemical Center
University of Lund
1984



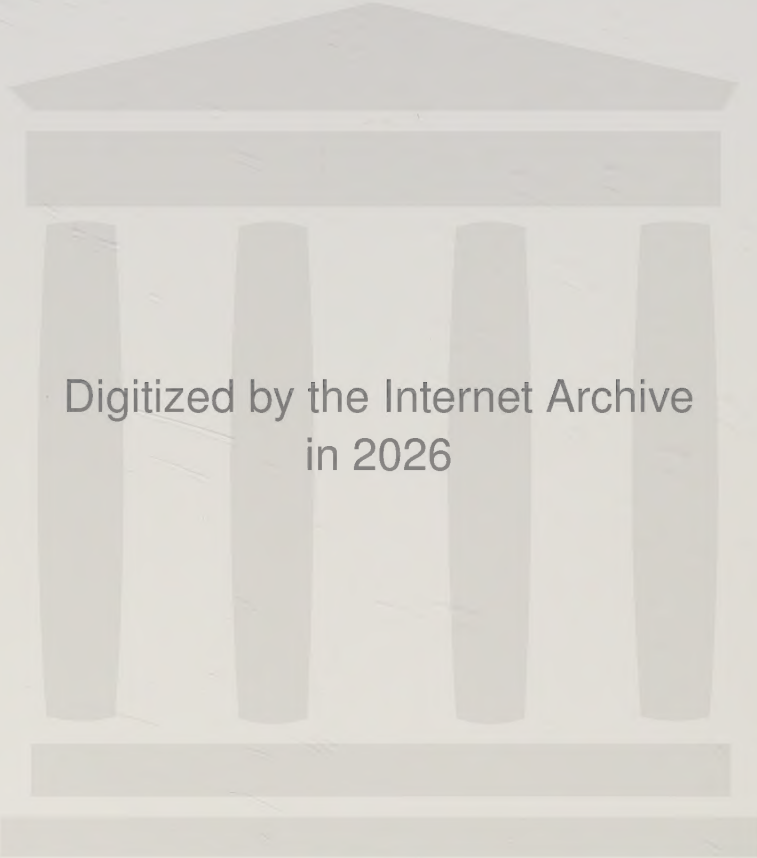
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on the conversion capacity of Pseudomonas spp.

Inge Nilsson
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Akademisk avhandling, som för avläggande av
teknologie doktorsexamen vid tekniska fakulteten
vid Lunds Universitet kommer att offentligen
försvaras å Kemicentrum, hörsal C, Sölvegatan 39,
Lund, onsdagen den 30 maj 1984, kl 10.00.

Organization LUND UNIVERSITY Department of Applied Microbiology, Chemical Center, University of Lund, PO Box 740, S-220 07 Lund, Sweden		Document name DOCTORAL DISSERTATION	
		Date of issue May 1984	
		CODEN: LUTKDH/(TKMB-1005)/206/(1984)	
Author(s) Inge Nilsson		Sponsoring organization Biotechnology Research Foundation National Board for Technical Development	
Title and subtitle Influence of entrapment and surface adsorption on the conversion capacity of <u>Pseudomonas</u> spp.			
Abstract Immobilization of whole cells is a technique which has been rapidly expanding in recent years. The immobilized cells are often subject to conditions very much different from those prevailing in suspension and thus the conversion capacities can change significantly. Alginate entrapped <u>Pseudomonas denitrificans</u> cells were used in denitrification of water and the gel immobilization proved a gentle method giving viable denitrifying preparations. Limited mechanical integrity and diffusional restrictions however hamper the use of this technique in large scale denitrification. The adsorption of microorganisms to inert surfaces is a simple and cheap immobilization technique. Attached biofilms can advantageously be used in continuously operating reactors, where dilute substrates can be processed at high flow rates. It was shown for <u>Pseudomonas putida</u> and <u>P. denitrificans</u> that dilution rates above μ_{max} of the cells change the adhesive properties of the organisms and enhance biofilm formation rates. When phenol was aerobically degraded by <u>P. putida</u> in a continuous biofilm culture, the area and configuration of the inert surfaces available for attached growth influenced the productivity. This fixed film reactor was also applied to microbial denitrification of water by <u>P. denitrificans</u> and proved to be a suitable alternative to the reactor holding gel entrapped cells.			
Key words Immobilization, alginate gel, denitrification, adsorption, biofilm, phenol degradation, biofilm build-up, <u>Pseudomonas</u>			
Classification system and/or index terms (if any)			
Supplementary bibliographical information			Language English
ISSN and key title			ISBN
Recipient's notes		Number of pages 206	Price
		Security classification	

Distribution by (name and address) Dept Applied Microbiology, Chemical Center,
PO Box 740, S-220 07 Lund, Sweden

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Date April 7, 1984

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A LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

- I Nilsson I, Ohlson S, Häggström L, Molin N and Mosbach K (1980)
Denitrification of water using immobilized Pseudomonas denitrificans cells
Eur J Appl Microbiol Biotechnol 10:261-274

- II Nilsson I and Ohlson S (1982)
Columnar denitrification of water by immobilized Pseudomonas denitrificans cells
Eur J Appl Microbiol Biotechnol 14:86-90

- III Molin G, Nilsson I and Stenson-Holst L (1982)
Biofilm build-up of Pseudomonas putida in a chemostat at different dilution rates
Eur J Appl Microbiol Biotechnol 15:218-222

- IV Molin G and Nilsson I (1983)
Effect of different environmental parameters on the biofilm build-up of Pseudomonas putida ATCC 11172 in chemostat
Eur J Appl Microbiol Biotechnol 18:114-119

- V Nilsson I and Dostálek M
Estimation of the biofilm formation ability of Pseudomonas putida in discrete samples from continuous culture
Submitted

- VI Molin G and Nilsson I
Sand administration as an instrument for biofilm control of Pseudomonas putida ATCC 11172 in chemostat cultures
Submitted

VII Molin G and Nilsson I

Degradation of phenol by Pseudomonas putida ATCC 11172 in chemostat cultures at different degrees of biofilm activity

Submitted

VIII Nilsson I

Biofilm build-up and microbial denitrification of high nitrate water by surface adsorbed Pseudomonas denitrificans cells

Submitted

B INTRODUCTION

Surface adsorption of microbial cells is a phenomenon frequently experienced in nature. The development of attached biomass can be observed in very diverse natural environments, e.g., on soil surfaces, in marine environments and on animal tissues. Early evidence for bacterial adhesion to surfaces was presented by, for example, Pasteur in 1864. The deliberate use of spontaneously formed biofilms in "industrial" applications such as vinegar production, bacterial leaching of ores and waste water treatment has been manifested for quite a few years. Thus, it is somewhat surprising that the cell immobilization techniques have not, until recently, gained significant interest. The area of immobilized whole cells has expanded only within the last 5 to 10 years, via the development of immobilized enzyme processes. The development started in the 1960s by e.g. the adsorption of bacterial cells to ion exchange resins and the entrapment of lichen cells in polyacrylamide gels.

A wide variety of immobilization techniques, to varying degrees of sophistication, have been developed in the last years. However, not all of these are, for several reasons, suitable for potential large scale applications. The immobilization techniques cover methods from microcapsule and lattice entrapment, covalent cross linking etc to the "rather uncomplicated" adsorption of growing or non-growing cells onto an inert surface. The choice of preference in a specific situation is highly dependent on factors like mass transfer requirements, need of aseptic products, molecular weight of substrate and product and type of active enzymic systems required.

One interesting area is the "borderline" between suspended and immobilized cultures, where growing cells are allowed to spontaneously adsorb onto an inert surface. The cells, thus immobilized, can advantageously be utilized under either growth conditions or as resting cells in continuously operating reactors. Many factors influence the adsorption process, the activity of the biofilm and the decay of the attached

biomass.

C OBJECTIVE OF THE PRESENT INVESTIGATION

Some years ago immobilized cells were mostly applied to non-cofactor requiring one step enzymic conversions. At a later stage more complex reaction patterns were utilized but due to diffusional limitations most applications have concerned anaerobic conversions. Most processes have dealt with high-price products produced in limited volumes. The use of immobilized cells in bulk product industries has been limited. As described below immobilized cell systems show both merits and disadvantages compared with "conventional" fermentation.

Microbial denitrification, which is one model system investigated in this study was chosen for two major reasons. Firstly, high nitrate concentrations are today frequently found in surface and ground waters and there is a need for simple, cheap and efficient methods of reducing the nitrate in these polluted waters. Secondly, microbial denitrification is a process that exhibits many features generally important in the evaluation of immobilized cell techniques. Fully viable cells are required and it is an anaerobic process. As such it does not need oxygenation, but gaseous products influencing the immobilized system, are produced. The accumulation of intermediate products such as nitrite, partly due to diffusional limitations, is another important factor. The process per se does not require cell proliferation, which makes it suitable for immobilization.

The objective of the first part of this study was to investigate alginate immobilized Pseudomonas denitrificans cultures in microbial denitrification of potable water and demonstrate some advantages and drawbacks exhibited by this immobilization technique (Papers I and II).

The adsorption of non-viable or viable (growing or non-growing) cells onto inert surfaces is probably the most simple



immobilization technique and it is well known in, e.g., waste water treatment. In spite of minimized diffusional limitation, drawbacks like low cell loading capacity, no protection against mechanical forces etc have hitherto limited the use of adsorption as an immobilization technique. As previously stated the spontaneous adsorption of growing cells onto surfaces offers process possibilities in the field between immobilized and suspended cell cultures.

The second part of this study concerns biofilm build-up in continuous reactors and the objectives were several. Firstly, to investigate a few general factors possibly influencing the rate of Pseudomonas putida biofilm formation (Papers III to VI). Secondly, to apply the concept of fixed biofilms to suitable continuously operating model systems. Phenol degradation by Pseudomonas putida and microbial denitrification by Pseudomonas denitrificans were studied as they are of potential interest in water and waste water treatment. They also exhibit very different characteristics important in the evaluation of biofilm reactors. As previously described, microbial denitrification is an anaerobic non-growth related process with gaseous products whereas phenol degradation is a highly aerobic process converting the carbon source mainly to microbial cell mass. Wall growth in a continuous reactor often influences the possible flow rates, resistance to environmental changes etc. The arrangement of the inert surfaces in a reactor is also an important issue affecting oxygen transfer. These factors are discussed in Papers VI and VII. Finally, the build-up of a pure culture denitrifying biofilm and the subsequent treatment of high nitrate water is described (Paper VIII), and this immobilization technique is compared with "conventional" alginate immobilization.

D LITERATURE BACKGROUND

IMMOBILIZATION TECHNIQUES

The dynamic development of research in the field of immobilized cells has resulted in excellent reviews covering many aspects on immobilization techniques. Examples of recent reviews are given in Table 1.

Table 1. Examples of review articles on cell immobilization

Abbot BJ	1977	Messing RA	1981
Jack TR and Zajic JE	1977a	Bucke C	1983
Durand G and Navarro JM	1978	Birnbaum S et al.	1983
Klein J and Wagner F	1979	Mattiasson B	1983
Kolot F	1980	Nilsson K	1983
Atkinson B et al.	1980	Ramstorp M	1983
Kolot F	1981a,b		

With reference to these reviews, this background will merely give a very brief introduction to some of the whole cell immobilization techniques used and discuss some advantages and drawbacks connected with these methods.

Prior to the description of immobilization techniques it may be appropriate to compare the immobilized cell system with a conventional fermentation process in order to consider what advantages can be gained. Some of the merits of the immobilized systems are mentioned below:

- o increased cell densities can give higher reaction rates and reduce the required size of the reactor.
- o continuous operation is often possible.
- o high flow rates are possible without washout of microbial cells.
- o product up-grading is facilitated.
- o increased protection of the cells against environmental changes.

As indicated there are a number of advantages in the use of immobilized whole cells but it is important to realize the drawbacks exhibited. Some of these are:

- o the immobilization method chosen may be detrimental to the cellular activity
- o diffusional limitations hamper the mass transfer
- o extra cellular enzyme activities are lost
- o intra cellular products are often inaccessible

Probably the most important disadvantage is the diffusional limitation which decreases the transport of substrates. This effect is pronounced when substrates of low concentration or low solubility are utilized. It also contributes to the accumulation of possibly toxic or inhibitory products. However, it is important to be aware that the various immobilization techniques to a very different extent, suffer from these drawbacks. This will be further discussed below.

If immobilized cells are to be used on an industrial scale certain criteria which to some extent limit the number of procedures applicable, have to be met:

- o immobilization procedure should be simple. Inexpensive materials are to be used unless an unsophisticated regeneration process can be applied.
- o immobilization procedure should not involve toxic components liable to harm plant operators or product users.
- o immobilization procedure should be mild. Unless single enzyme activities are to be utilized, cell viability ought to be retained in order to provide possibilities for regeneration of the immobilized preparation.
- o the half-life of the immobilized preparation should be long. This is important when considering the cell activity as well as the mechanical stability of the preparation.
- o the activity of the immobilized cells should be high. This means minimal diffusion restrictions combined with strong binding of the cells in order to prevent cell leakage into the product stream.

- o the immobilization procedure should be inexpensive.
- o the immobilized cell system should be able to handle commercial substrates.

It must be stressed that immobilized cells will always be compared with alternatives such as conventional fermentation (in batch or continuously operated), soluble or immobilized purified enzymes.

The potential application fields of immobilized microorganisms are e.g. production of chemicals, detoxification processes and analytical systems. It is, of course, rather difficult to establish which processes are today run on an industrial scale. A summary of industrially established processes and potential ones is given by, e.g., Birnbaum et al. (1983) and Bucke (1983). In general, systems most easily brought onto an industrial scale are those where only small volumes of high-price products are to be produced. Other potential areas are processes where biochemical systems normally not co-existing are brought together to give bioconversion opportunities previously not experienced.

The following gives a brief introduction to a few commonly used immobilization techniques.

Adsorption. As adsorption is a comparatively inexpensive immobilization procedure, it is suitable particularly for large scale processes involving inexpensive substrates and products. The adsorption method is also very applicable to, and sometimes necessary for, sensitive systems, e.g., mammalian cells. The latter systems will, however, not be discussed further. Cell adsorption and biofilm build-up are complex phenomena influenced by many factors. Three major variables affecting the microbial adsorption onto solid surfaces can be distinguished; the character of the micro-organism, the substratum and the environment. The importance of these and other variables is illustrated in the next

section.

Covalent coupling. This method is mostly used for immobilization of enzymes to various supports. It often involves chemicals having detrimental effects on living cells, which makes the method unsuitable in systems requiring viable cells. One example in the field of covalent attachment is the production of urocanic acid from L-histidine by non-viable Micrococcus luteus cells coupled to carboxymethyl cellulose via carbodiimide (Jack and Zajic 1977b). Another is the coupling of Zygosaccharomyces lactis to hydroxyalkyl-methacrylate (Jirku et al. 1980). It could also be mentioned that the procedures used are relatively elaborate.

Encapsulation. This is based on the confining of cells within semi-permeable membranes, through which small molecules can diffuse. The use of organic solvents deleterious to the viable cells may limit the use of this method. One example is given by Mohan and Li (1975) using liquid-surfactant membrane encapsulated Micrococcus denitrificans cells for the denitrification of water.

Another method in the field of "encapsulation" is the confinement of cells within e.g. hollow fibre membranes. Such reactors have been used for the production of urocanic acid (Kan and Shuler 1978) and denitrification of water (Mattiasson et al. 1981).

Utilization of aqueous two-phase systems, reviewed by Mattiasson and Hahn-Hägerdal (1983), is again another technique in this field.

Entrapment. This is by far the most frequently used method for cell immobilization. The procedure is quite simple. Cells are mixed with monomers or polymers and after gel formation the cells are confined into a polymer network. Problems with cell leakage are reduced compared with, e.g.,

adsorbed cell systems. Entrapment is generally a gentle method and if the immobilization method is properly chosen, the viability of the cells is often retained. There are certain disadvantages associated with the entrapment procedures. One is that the preparations often show diffusional limitations. This drawback can, to some extent, be circumvented by modifying the particle and pore size of the biocatalyst preparation (Klein et al. 1983). This increase in diffusion capacity has to be put against the possibility of increased cell-leakage, particularly in systems requiring viable cells. The pore size of the biocatalyst also determines the maximal molecular weight of the substrate (Tanaka et al. 1984).

The polymers commonly used for entrapment are either synthetic or derived from natural sources. The most important synthetic substances are acryl polymers (polyacrylamide) and polyurethanes. The toxic effects of the monomers and of the polymerization conditions can possibly be reduced by the use of prepolymers. Only the final step is completed in the presence of microbial cells.

The natural polymers used in entrapment are either proteins like collagen or carbohydrate based like agar, agarose, alginates, K-carrageenan etc. At least two methods are used to create the gel lattice: thermal and ionotropic gelation. Agar is the classical example of thermal gelation. K-carrageenan shows a similar behaviour but requires a continuous presence of, e.g., potassium as well. Alginate and chitosan are examples of polymers forming gel lattices in contact with certain counter ions like Ca^{2+} , Al^{3+} (alginate) or PO_4^{3-} (chitosan). However, this gel formation is reversible and the presence of complexing agents solubilizes the gel. This problem can be circumvented by using certain crosslinking procedures involving glutaraldehyde, polyethyleneimine etc (Birnbaum et al. 1981). Then, of course, some of the gentleness of these methods is lost. Calcium alginate is probably the most widely used entrapment gel in research. It is cheap and very easy to use. However, it is not ideal when e.g.

high mass transfers are required and it is definitely unsuitable when chelating compounds are involved.

MICROBIAL ADHESION TO SURFACES AND BIOFILM FORMATION

Microbial activity within attached biofilms plays an important role in many fields e.g. waste water treatment operations and fermentation processes. Biofilms are also often responsible for detrimental effects such as increased frictional resistance, reduced heat transfer capacity, accelerated corrosion in heat exchanger and water conduits etc. It has been suggested (Bryers and Characklis 1982) that biofilm development is the result of several transport phenomena and biological processes. The suggested contributing processes are:

- o adsorption of organic material onto the surface
- o transport of microorganisms to the surface
- o microbial adhesion to the surface
- o growth of attached microorganisms
- o detachment of established biofilm

Exposure of a "clean surface" to a fluid containing nutrients, organic material etc. results, within minutes, in an organic monolayer conditioning the inert surface. The transport of microorganisms to the surface is of course dependent on e.g. the shearing forces produced by the liquid flow rate. The microbial adhesion is often considered as a two-stage process; first an instantaneous reversible adhesion followed by an irreversible attachment, sometimes mediated by polymer bridging (Marshall et al. 1971). The growth of the biofilm is quite often a combination of cellular reproduction and production of polymeric material. The final process, biofilm detachment, is dependent on for instance the shearing forces.

General concepts

Before discussing different factors influencing the adhesion processes, it may be appropriate to briefly discuss a few physico-chemical aspects of adhesion to surfaces. Microbial cells are not "ideal particles" to study as they do not have a simple geometry and they do not have a uniform molecular composition. However, it is still interesting to describe the interactions between charged particles, like microorganisms, and macroscopic surfaces and how the presence of macromolecules influences this interaction.

The major forces operation in particle deposition are van der Waals forces, double-layer electrostatic interactions, steric and bridging interactions (Tadros 1980). The net interaction between a charged particle and a macroscopic surface can be described in terms of the interaction free energy (G_i) as a function of separation distance (d). The DLVO-theory states that the interaction comprises two additional terms: G_A due to van der Waals forces and G_E due to the overlap of electrical double layers associated with the surfaces (Rutter and Vincent 1980). The schematic appearance of the interaction free energy (G_i) versus separation distance (d) is shown in Figure 1 at different electrolyte concentrations for a charged particle approaching a macroscopic surface of the same sign.

The essence of Figure 1 is that at intermediate electrolyte concentration there are two minima, one primary representing irreversible attachment and one secondary representing the reversible state of adhesion. These long-range interactions are of importance to microbial deposition on surfaces.

The presence of macromolecules in solution or at the interfaces can drastically change the adsorption behaviour. Firstly the adsorption rate constants can be reduced due to increased viscosity. Secondly the G_A and G_E values may be changed and thirdly a steric interaction influences the system when both surfaces are covered with macromolecules (Rutter and Vincent 1980).

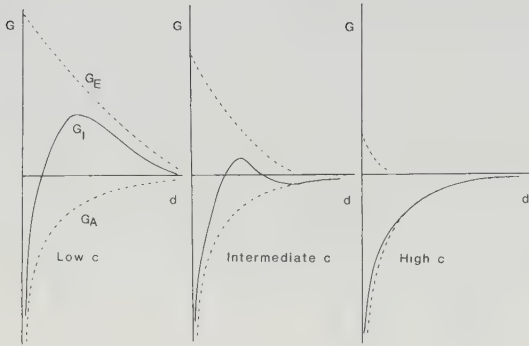


Figure 1. Interaction free energy versus separation distance at different electrolyte concentrations for a charged particle approaching a macroscopic surface of the same sign (Rutter and Vincent 1980).

Finally if one of the surfaces is not covered with polymer, bridging is liable to occur. Long range forces are important to particle deposition, and short range forces like chemical bonds, dipol interactions and hydrophobic bonding are important in adhesion strength (forces necessary to detach adsorbed particles). Once the particle is sufficiently close to the surface the short range forces are of substantial interest.

Again it must be emphasized that these ideal model interactions are for several reasons very inadequate to biological systems. Microbial cells and other surfaces are rough, implying that contact is established at many points. Certain cell types are deformable which means that spherical particles are not at hand. The complex composition of the cell surface allows many types of interactions simultaneously. Macromolecular substances produced by microorganisms form conditioning layers on substrata in order to facilitate the attachment process.

Another approach to the adhesion phenomena often considered is to investigate the "wettability" of a solid surface (Fletcher et al. 1980). This can be estimated by placing a drop of liquid on a horizontal solid surface.

By measuring the contact angle for a series of homologous liquids, plotting the surface tensions of these against the cosines of the contact angles and extrapolating to $\cos \theta = 1$ a surface tension called the critical surface tension for wetting (γ_c) is obtained (Zisman 1964). These and similar methods have been further developed (e.g. Fletcher and Marshall 1982). According to the γ_c - values solid substances are classified into different groups, e.g. high energy surfaces like glass, mica and cellulose and low energy surfaces such as teflon. Examples of surface tensions and surface energies for different liquids and solid substances are given by Gerson and Zajic (1979). It is important to realize that only the outer layers are important for the surface properties. An adsorbed monolayer of water on a high energy surface means that the γ_c , or rather γ_s (surface energy) value rapidly decreases. This also means that the addition of surfactants etc. may drastically change the adhesive properties.

Rather few measurements of surface energies of living cells have been made, but a summary of such values is given by Gerson and Zajic (1979). They also compiled data from different sources indicating that the adhesive strength increases up to the point where the surface free energies of substrata and adhesive organisms are equal, and then decreases. This is much in accordance with the results found by Dexter et al. (1975), showing with electron microscopic studies that the bacterial attachment greatly increases with increasing critical surface tension. These reports confirm that the description of substrata in terms of surface energy is quite important in the investigation of microbial attachment. As stated by Fletcher and Marshall (1982) it is rather easy to characterize clean substrata but the apparent character-

ristics change very rapidly when the surfaces are immersed into aqueous solutions containing bacteria and dissolved substances.

Factors influencing microbial adhesion

A study on the effects of different factors influencing the adsorption process can be performed with respect to the character of the microorganism, of the substratum or of the environment.

Character of the microorganism: Bacterial strains differ considerably in the chemistry and structure of the cell envelope. The walls of all bacteria, except the Archaeobacteria, contain peptidoglycans as a main structural component. The cell walls of gram positive bacteria also contain other polymers like teichoic acids and certain polysaccharides. The cell wall of gram negative organisms is more complex. In addition to a rather low content of peptidoglycan there is an outer membrane containing proteins, phospholipids and lipopolysaccharides. Except for the cell wall and the outer membranes, there are other wall associated structures in contact with the environment. The capsules and slime layers are to some extent artificially divided into two groups; slime layers and microcapsules which are difficult to visualize, and macrocapsules visualized after negative staining. As shown by Ward and Berkeley (1980) a wide variety of organisms produce capsules and slimes of different composition, usually various polysaccharides. Other wall associated structures are pili (fimbriae) and flagella.

What structures are then involved in adhesion processes? It was found by Hermansson et al. (1982) for Salmonella typhimurium and Serratia marcescens that reduction of the lipopolysaccharides of the cell wall changed the hydrophobicity. Similar results, showing that a loss of oligosaccharide components of the lipopolysaccharides in E. coli was accompanied by an increase in the affinity for hydrophobic surfaces,

were demonstrated by Rosenberg et al. (1980). Bacterial fimbriae are also important to microbial adhesion. Hermansson et al. (1982) showed that fimbriated S. marcescens were less hydrophobic than non-fimbriated counterparts. Gibbons et al. (1983) demonstrated that non-hydrophobic mutants of Streptococcus sanguis, defective in synthesis of fimbriae, had a greatly diminished capacity for attachment.

Bacterial flagella give the organisms motility and thereby a possibility of responding to chemotactic stimuli, which can be of importance in connection to nutrient accumulation on surfaces.

The importance of capsular material and slime layers in bacterial adhesion has frequently been described. The role of microbial exopolysaccharides for microbial adhesion in aqueous systems has recently been reviewed (Sutherland 1983). The development of staining procedures (e.g. ruthenium red staining) has permitted the study of polysaccharides in electron microscopy. It was found by Fletcher and Floodgate (1973), in a study of an unidentified gram negative mobile rod shaped organism, that an acidic polysaccharide was present on the suspended bacteria and appeared to assist adhesion. Bacteria attached to the surface produced a second type of polymer, different in appearance. In the selection of attachment mutants of Pseudomonas fluorescens in continuous culture, Pringle et al. (1982) indicated the enrichment of two classes of mutants with surface characteristics and extracellular polymer production fundamentally different from the wild type. Further information on microbial exopolymers can be found in a recent review by Geesey (1982). Organisms exhibiting holdfasts, like e.g. the Caulobacter group, are also important in microbial attachment to surfaces.

Some characteristics of the cells which influence attachment are dependent on the age of the culture. Such characteristics

are, e.g., cell motility and quantity and quality of cell surface polymers. It was shown by Fletcher (1977), for a marine pseudomonad, that the largest proportion of motile cells was found in log-phase cultures and it was suggested that the decline in attachment with culture age could be due to a reduction in cell motility. Furthermore, the author also stated that the change in the production or secretion of adhesive polymers with culture age influenced the adhesion. Duddridge et al. (1981) concluded that, in static adhesion studies of Pseudomonas fluorescens, cells in stationary phase appeared "stickier" than did cells in logarithmic phase when adhering to metal surfaces.

Character of the substratum: The second main component in the adhesion process is the substratum. An enormous variety of solids have been used in adsorption studies. An extensive review has been provided by Daniels (1972) and more recent one by Kolot (1981b). A few more examples are given in Table 2. Two major factors are of importance when evaluating the substrata: The physico-chemical character and the geometry. Firstly, the physico-chemical character, often given in terms of substratum wettability. A number of investigations concerning attachment of marine bacteria have shown contradictory results. Some in situ experiments involving a range of substrata have demonstrated that attachment to hydrophilic (high-energy) surfaces like glass and metals was more rapid (Dexter 1979) whereas laboratory studies involving marine pseudomonads showed that these organisms colonized hydrophobic (low-energy) surfaces more rapidly (Fletcher and Loeb 1979). These apparently conflicting reports are probably explained by the fact that different genera or even strains of bacteria exhibit varying attachment to substrata. No generic pattern was found when the attachment of fresh water bacteria was tested on different substrata (Pringle and Fletcher 1983). However, a majority of organisms attached better to hydrophobic surfaces. Another explanation to the contradictory reports may be that substrata exposed to, for exam-

ple seawater, rapidly adsorb a "conditioning film" of organic molecules which drastically changes the character of the surface (Baier 1970). Fletcher (1976) showed that different proteins impaired the attachment of marine pseudomonads to polystyrene dishes. By depositing fatty acids onto a solid substratum, Larsson and Glantz (1981) found that nonpolar surfaces exhibited the same adhesiveness as did the reference metal surfaces. However, the adhesion was completely inhibited on surface layers having a high negative charge.

Table 2. Examples of solids used for microbial attachment

Solid	Organism	Reference
Vermiculite	<u>Zymomonas mobilis</u>	Bland et al. (1982)
Aluminium	<u>P. fluorescens</u>	Bott & Miller (1983)
Metal surfaces	" "	Duddridge et al. (1981)
Cordierite	<u>Acetobacter aceti</u>	Ghommidh et al. (1981)
Kaolinite	-----	Jian et al. (1983)
Polyphenylenoxide	<u>Kluyveromyces lactis</u>	Jirku et al. (1980)
Ion exch. resins	<u>Zymomonas mobilis</u>	Krug & Daugulis (1983)
Cellulose	<u>Sacch. cerevisiae</u>	Lueng et al. (1983)
Wood chips	" "	Moo-Young et al. (1980)
Diatomite	" "	Moo-Young et al. (1980)
Tuff	" "	Parascandola et al. (1983)
PVC-granules	<u>P. aeruginosa</u>	Tengerdy et al. (1981)
Glass	Mixed culture	Pedersen (1982)
Asbestos	<u>Aureobasid. pullulans</u>	Takahashi et al. (1981)
Corn stovers	<u>P. aeruginosa</u>	Vossoughi (1982)
Earthenware	Mixed culture	Vossoughi (1982)

The second factor of importance is the geometry of the surfaces. This is even more conspicuous when the attached films are to be used in different reactors and will be further discussed in the following section. The pore size of a porous material is important to the accumulation of adsorbed microbes. Investigations by Messing and Oppermann (1979) and Messing et al. (1979) revealed that a porous inorganic carrier

should have a pore diameter of one to five times the largest dimension of the cell. It was further stated that the required pore size is dependent on the mode of reproduction of the organism. Sporeforming organisms and microbes exhibiting mycelial growth tend to accumulate more efficiently on the porous surface if the pore size is one to sixteen times the dimension of the fungal spore.

Character of the environment: The interaction between micro-organisms and inert surfaces is, as described above, dependent on the character of the cell and the substratum but many environmental factors are of equal importance.

The concentration of cells in the suspension influences the number of collisions with the inert media and thus possibly the attachment. Fletcher (1977) reported that when a non-growing marine pseudomonad was exposed to polystyrene, the number of attached cells was greatly influenced by the cell concentration. This effect was more pronounced for log phase cells compared with stationary or death phase cells. Similar results were shown by Duddridge et al. (1981) when exposing Pseudomonas fluorescens cultures to stainless steel surfaces.

The time of exposure naturally also influences the number of initially adsorbed organisms. This was demonstrated by Fletcher (1977) and Duddridge et al. (1981).

The temperature, which has a great influence on metabolic activities and reaction rates, also affects the attachment processes. A decreased number of attached bacteria at low temperatures, under non-growth conditions, were found in two investigations by Fletcher (1977) and Duddridge et al. (1981). Three explanations were suggested for this decrease in attachment at low temperatures ($3-5^{\circ}\text{C}$ compared to $20-30^{\circ}\text{C}$). Firstly, the increase in medium viscosity when the temperature is lowered. Secondly, low temperatures disfavour chemisorption and physical adsorption of solutes. Thirdly, a low temperature can influence attachment processes by affecting the cell physiology.

A great many characteristics of the cell surface and the substratum surface are influenced by the pH-value of the medium. Surface charge, surface potential, electrical double layers as well as bacterial flocculation processes and attachment to particles like ion exchange resins are affected by pH. It was demonstrated by Wilkinson and Hamer (1974) that in a mixed culture, dominated by a Pseudomonas sp, the biofilm build-up was significant at a pH above 5.8 while no film developed at pH-values below 5.5. The influence of pH on adsorption to ion exchange resins has been thoroughly examined by e.g. Wood (1980). Work on the interaction of microorganisms with ion exchange resins is mostly concerned with anion exchangers. It has been suggested that the adsorption of bacteria is mediated by the carboxylic groups of cell surface proteins. Wood (1980) concluded that the interactions between ion-exchange resins and microorganisms can be divided into four groups according to the pH at which they occur: 1) Non-specific adhesion which is most abundant close to the iso-electric point of the microorganism. 2) Adhesion to anion exchange resins at pH-values above the iso-electric point of the cells. 3) Adhesion to cation exchange resins at pH-values below the iso-electric point of the cells. 4) Salt dependent adhesion to cation resins at pH-values above the iso-electric point of the cells. Concerning other substrata, Duddridge et al. (1981) found that the attachment of Pseudomonas fluorescens to stainless steel under static conditions was not affected at pH-values from 4.5 to 7, but decreased significantly at higher pH.

A most interesting factor involved in microbial attachment is the nutrient availability. Brown et al. (1977) showed in nitrogen or carbon limited chemostats with mixed cultures, that a biofilm developed only in the carbon limited culture. These results are similar to those reported by Marshall et al. (1971), showing that attachment of a Pseudomonas sp to glass in a marine environment was inhibited at an excess glucose concentration in the water. It is quite clear that attachment to surfaces is often associated with growth at low nutrient levels e.g. in natural environments. Another interesting

aspect shown by Dawson et al. (1981) is that starvation conditions enhance the rate of adhesion. A marine Vibrio sp produced "dwarf-cells" on starvation and these cells showed an enhanced adhesion to siliconized glass, an effect related to a production of a bridging polymer.

As shown in a review by Fletcher (1980) cation concentrations in the media are important to adhesion in several ways: indirectly by influencing cell physiology, directly by cation accumulation on surfaces forming electric double layers. Other "environmental" factors of importance to the attachment properties are e.g. dilution rate and shearing forces. These will be further discussed in the following section.

BIOFILMS IN CONTINUOUS REACTORS

Discussion has hitherto been limited to factors influencing the microbial adhesion to surfaces in general. Attached biofilms can advantageously be used in continuous reactors. Early examples are waste water treatment and production of vinegar. Important parameters connected with the use of biofilm reactors are e.g. reactor configuration, choice of inert substratum, geometry of substratum, physiological effects on attached microorganisms and control of biofilm thickness.

Reactor and support configuration

Several reactor models are possible when attached biomass is to be used (Atkinson and Mavituna 1983). In principal, one can distinguish two systems. Firstly, reactors where the liquid flows across the biofilm surface and secondly reactors where the solid surfaces holding the biomass move within the liquid phase. Examples of the first type are trickling filters, packed bed reactors and ordinary fermentation vessels. The second group involves e.g. rotating biological contactors and fluidized bed reactors. There are numerous examples of the use of such reactors in both waste water treatment (Christensen and Harremoës 1977) and conventional fermentation (Atkinson and Fowler 1974, Gainer et al. 1980).

As previously indicated the choice of inert support material is important. Murray and van den Berg (1981) demonstrated for example that the development of methanogenic fixed films was three times faster on fired clay than on PVC plastics or etched glass. This was explained partly by the rough porous surface of the clay, partly by the presence of minerals (e.g. iron) known to stimulate methanogenesis and microbial growth. In reactors containing suspended support particles the geometry of the particles is as important as the material itself. If smooth spherical particles are utilized the abrasive forces may well prevent the biofilm formation completely. The use of porous materials such as stainless steel wire spheres (steel wire, knitted and crushed into a sphere), polypropylene toroids, reticulated polyester foams etc allows microbial growth within the particle (Atkinson et al. 1979, 1980 a and b). Once filled with biomass, the particle to particle collisions maintain the amount of attached cells on this level as growth on the outside is prevented. It has been suggested that particles of any size and shape can be "filled" with almost any organism. The adhesive capacity of different organisms varies, but attachment mutants can be selected in continuous culture. It was shown by Pringle et al. (1983) that mutants of Pseudomonas fluorescens with increased attachment to solid surfaces were produced in a continuous culture. The selection pressure used was foam production which fractionated the wild type cells from the fermentor.

Biofilm production and control

From an economical point of view it is important to 1) have a fast production of biofilm and 2) maintain the thickness of the biomass layer on a desired level. A few ways of accelerating attachment and biofilm formation have been presented. One is to subject the organism to starvation (Dawson et al. 1981). Similar effects were demonstrated by Häggström (1984), when exposing Clostridium acetobutylicum cells to low nutrient media in continuous culture. Another way to promote attachment is to precondition the inert surfaces with synthetic or natural polymers (La Motta et al. 1982). It was shown that the development of nitrifying films in waste water treatment

accelerated when the surfaces were precoated with synthetic cation polymers. The biofilm growth was even further increased when naturally produced biopolymers were used as the precoat. It was suggested that a practical way of preconditioning surfaces prior to adhesion of a "non-adherent" organism is to develop a fast growing microbial film by using an adhesive micro-organism.

Measurements of microbial films in different biological reactors have shown values covering a considerable range, from 3×10^{-4} to $0.4 \text{ g wet weight/cm}^2$ (Atkinson and Fowler 1974). The thickest films often develop when mixed cultures are used e.g. in waste water treatment systems. When "non-adhesive" pure cultures are grown in stirred reactors the biofilm thickness is in the range 0.001 to 0.01 mm, often only a monolayer of organisms. The biofilm thickness is important, as diffusional limitations hamper the mass transfer to the inner layers of the biofilm if the thickness is more than 0.1 to 0.2 mm. Results presented by Kornegay and Andrews (1968) and Tomlinson and Snaddon (1966), concerning substrate removal rates and removal of carbon in solutions with mixed cultures, indicated active film thicknesses of 70 - 100 μm . Measuring oxygen profiles in microbial slime at different glucose concentrations, it was concluded by Bungay et al. (1969) that the active film thickness is dependent on the substrate concentration of the medium. When the film exceeds the active thickness, regions with gaseous products, gas bubbles, lysing cells etc develop close to the inert surface. This in turn gives random sloughing of biofilm which is a disadvantage to a continuous process and implies that it is often desirable to control the biofilm thickness or, in some cases, eliminate the attachment completely. In e.g. a fluidized bed reactor excess biofilm may cause sedimentation or flotation of the inert media. Biofilm control can be achieved by mechanical means, e.g. scraping devices or administration of inert particles in a stirred reactor (Atkinson and Fowler 1974). In a fluidized bed the frequencies of particle collision are altered when the flow rates are changed, which in turn influences the abrasion.

It is possible to reduce biofilm formation by changing environmental conditions such as the pH-value. Atkinson and Fowler (1974) demonstrated that for a mixed culture of zoogloeal character, the biofilm formation decreased drastically when the pH-value was lowered from 7 to 5. It is obvious that such methods have disadvantages since the conditions required for inhibition of biofilm formation may be quite inappropriate for the fermentation process in question.

The administration of inert particles in stirred reactors in order to give a certain attrition is possible but it is most difficult to achieve a predetermined film thickness (Atkinson and Fowler 1974).

Physiological effects by surface adsorption

It is known that microorganisms attached to surfaces are metabolically more active compared with cells suspended in the surrounding medium, possibly due to nutrient accumulation at the surface (ZoBell 1943; Lange 1976). Immobilized cells generate and are subject to a micro-environment often different from the surrounding bulk phase. It is quite obvious that, in aerobic biofilm processes, the cells often operate under oxygen deficiency. The changes in growth physiology and metabolism previously reported for immobilized microorganisms must be considered in the light of the different environmental conditions prevailing within the biofilm and in the bulk suspension.

It was reported by Navarro and Durand (1977) that yeast cells adsorbed onto porous glass showed a higher specific oxygen consumption rate compared with free cells. Similar results were presented by Marcipar et al. (1979) who suggested that the increase in oxygen consumption rate of the Saccharomyces cerevisiae cells adsorbed onto a ceramic material, was due to increased availability of nutrients and production of a protective micro-environment. On the other hand Bandyopadhyay and Ghose (1982) reported decreased oxygen uptake and carbon dioxide evolution rates of yeast cells adsorbed onto various

carriers. They also found an increase in the growth rate of the immobilized cells, suggesting a different maintenance requirement compared with the free cells. A reduction in generation time has also been reported by other researchers (Navarro and Durand 1980). Several authors (e.g. Navarro and Durand 1977, Tyagi and Ghose 1982) have described, using adsorbed cells, increased yields in ethanol production. It is likely that this is due to a change in the metabolic pattern. Another observation of increased maximal growth rate and cell yield of immobilized cells was reported by Hattori and Hattori (1981), when investigating Escherichia coli cells adsorbed onto anion exchange resin.

Detrimental effects of wall growth.

As described above the presence of surface-adsorbed micro-organisms is often advantageous in continuous reactors. High dilution rates can be employed without the risk of microbial wash-out and dilute substrates can more easily be processed. A system using attached biomass is likely to be more resistant to environmental changes.

However, there are also detrimental effects to be considered. Biofouling is a serious problem in heat-exchangers, water conduits etc. Decreased heat-transfer capacity, increased fluid frictional resistance, decreased efficiency of sensors and accelerated corrosion are a few examples of biofouling effects given by Characklis et al. (1982). Other important effects entirely disregarded in this study as they do not concern fermentation processes, are e.g. plaque formation on teeth and microbial attachment to animal tissues.

In laboratory investigations wall growth often hampers the usefulness of continuous culture (chemostat) as a research tool. Unexpectedly high values of maximum specific growth rate ($\mu_{\max}$) and maintenance coefficients reported, may well be due to wall growth in the reactor (Molin 1981: Cometta et al. 1982: Kuhn et al. 1980). Another interesting effect described by Dykhuizen and Hartl (1983) is the influence of wall growth on selection studies in chemostats. If selection

rate curves at some point change in slope, this is possibly due to the occurrence of wall growth.

Measurement of microbial films

Microbial attachment to surfaces and biofilm build-up are very complex phenomena. Progress in the understanding of microbial adhesion and biofilm formation to a great extent depend on the development of techniques applied to the study of such processes. It must be emphasized that the measurement method has to be chosen with regard to the objectives of the investigation. Naturally, different techniques are required when e.g. examining primary colloid attraction or when evaluating an established microbial film. This section will primarily describe some techniques commonly used in the examination of "fully" developed biofilms, their advantages and drawbacks and describe a few examples of application. More information can be found in the reviews by Characklis et al. (1982) Duddridge (1981) and Fowler and McKay (1980).

The measurement procedures of biofilm accumulation in different systems can be classified in two main groups, depending on the parameters studied: (i) direct measurement of biofilm quantity (ii) indirect measurement of biofilm quantity.

Direct measurement of biofilm quantity: There are two ways of direct measurement of biofilm quantity; biofilm thickness and biofilm mass. These values are naturally related to the biofilm density. One method of determining biofilm thickness was described by Characklis et al. (1982). The volumetric displacement of the biofilm was determined by immersing biofilm fouled tube samples into a displacement cell containing a water-surfactant solution. Various methods for determination of biofilm thickness, using equipment to locate the biofilm - fluid interface and biofilm-inert surface interface, have been described (Sanders 1966 and Norman et al. 1977). These methods are quite simple but certain drawbacks, like the need to remove test sections, drainage of the system or the need to make quick measurements due to film drying, hamper the use of

these methods.

The other direct measurement of biofilm quantity is the biofilm mass. The quantity of attached biomass can simply be estimated by drying samples to constant weight at increased temperatures ($> 105^{\circ}\text{C}$). The surfaces are then cleaned, dried and weighed once more. The relative error is of course influenced by the weight of biofilm compared with the total weight of the sample. The surface area available for attached growth is often known, which means that the biofilm density can be determined in terms of dry mass per unit of growth surface. On the other hand, if the biofilm thickness and volume have been measured, volumetric biofilm density (dry mass per wet volume) can be determined.

Indirect measurement of biofilm quantity: These methods can be divided into at least three subgroups: measurement of biofilm constituents, of microbial activity within the biofilm and of specific biofilm effects.

Considering the first group, biofilm parameters measured include: total attached polysaccharide, total organic matter, chemical oxygen demand and protein content.

As biofilms often contain large amounts of polysaccharides, quantitative methods for the determination of polysaccharide concentration are important. One method is based on the reaction of carbohydrate "reducing" ends (ketones or aldehydes) with non-oxidizing acids (Characklis et al. 1982). It is important to note that microbial exopolymers often contain non-sugar components influencing the amount of polysaccharide.

The second group of indirect biofilm measurements includes enumerations of total and viable cells, total cell count by fluorescence microscopy, determination of cellular components like ATP, lipopolysaccharides, total protein, muramic acids and poly- β -hydroxy butyrate. This group also includes methods for the determination of substrate removal rates. Results are

often reported as substrate removed per unit biofilm area, per unit biofilm mass or per unit biofilm volume.

Estimations of the microbial activity within a biofilm in terms of the total number of microorganisms, the distribution of organisms, variety of morphological types etc. can be performed through microscopic examination. It is possible to follow the in situ development of microbial attachment by immersing transparent surfaces, e.g. glass slides, into microbial suspensions or different waters. After removal of the slides the attached microorganisms can be counted, either directly or after a staining procedure. With smooth attachment surfaces the adhering cells can be fixed and stained in place and counted directly in e.g. a fluorescence microscope. When using irregular surfaces, however, the films must first be disrupted by sonication or other mechanical means, filtered onto a membrane, fixed, stained and then counted. This treatment, of course, gives a different estimate of the biofilm.

Electron microscopy is often advantageous to light microscopy as the high resolution can provide valuable information on the distribution of adhering bacteria in relation to the topographical appearance of the surfaces and on possible cellular appendages mediating the attachment processes (McCoy et al. 1981, Alleman et al. 1982).

Isolation and enumeration of viable cells from a biofilm by culture techniques involves homogenization procedures, dilutions and platings on nutrient media. By counting the colonies after incubation the microbial activity within the biofilm is estimated. These culture techniques can also be used in order to study the bacterial colonization with time or which organisms are predominant. These techniques are useful but it should be kept in mind that plate counts are somewhat unreliable due to microbial clumping and the number of viable cells is thus often underestimated. The choice of growth media and environmental conditions influence the type of organisms

isolated or enumerated.

One of the most widely used chemical measurements of biofilm activity is the determination of ATP content (Characklis et al. 1982). ATP is an interesting compound as it is only found in living organisms. This technique was used by, e.g., Harber et al. (1983) who developed a bioluminescence method for quantifying bacterial adhesion to polystyrene.

Finally, microbial activity in a biofilm can be measured by determining nutrient removal rates. Such methods have often been used to quantify the active thickness (Atkinson and Fowler 1974).

The third subgroup of indirect measurements of biofilm quantity covers determinations of fluid frictional resistance or heat transfer resistance in different reactors. Biofouling very much influences these properties.

The previous sections have dealt with methods used for the measurement of attached biofilm. Procedures dealing with the distraction or detachment of previously stabilized biofilms are also of importance. They can be divided in two groups; (i) a fixed distractive force is applied and the percentage of attached cells withstanding this is given. (ii) an increasing fluid shear force is applied and the minimum force resulting in detachment is given.

The first group involves methods applying different washing procedures, from very gentle "dippings" to the application of shearing forces removing the entire biofilm. Other detachment means are also used e.g. sonication (Tosteson and Corpe 1975). These methods involve problems with quantification of the distractive forces. These forces are often unevenly distributed on the surface, and this aggravate the comparison of data.

The other group of methods, the use of increasing shearing forces, suffer from the same disadvantages. These methods

are often designed to give a critical force required to remove a certain percentage of the attached cells during a pre-determined time. The shearing forces can be applied either by a liquid flow across the surface or by, e.g., rotating surfaces within a fluid.

One way to estimate a dynamic attachment process was demonstrated by Fowler and McKay (1980). They designed a radial flow growth chamber (Figure 2) in which test discs are placed. Cell containing fluid flows through the centre of the back disc at a constant flow rate and is radially distributed between the discs. This simultaneously provides a continuous range of shearing forces which must be overcome in order to give an attachment.

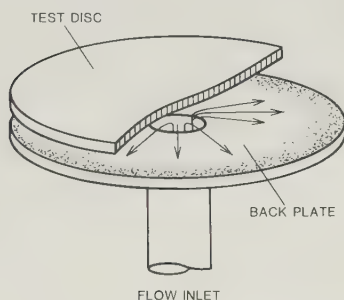


Figure 2. Radial flow growth chamber for adhesion measurements

This device, now commercially available as a "Cell Adhesion Measurement Module" (L.H. Engineering, England) can also be used in an alternative way. Attachment can take place under static conditions and when the flow is started the adhesive strength of the mature biofilm can be determined. It should be emphasized that this equipment can be run under sterile conditions.

Another way to achieve the same continuous range of shearing forces would be to rotate a disc in a culture fluid. However, swirling and sterility problems in the spinning system may hamper the use of this technique.

In conclusion, it is clear that a variety of methods is available for the measurement of mass and activity of an established biofilm. There are also methods for examination of the initial stages in the adhesion process. The choice of method is dependent on what kind of information is wanted, what kind of equipment and process is investigated, what aseptical conditions are required etc. It is important to realize the advantages and limitations of the different methods.

MICROBIAL NITRATE REDUCTION

A part of this work concerns aspects of the reduction of nitrate in potable water using entrapped or surface adsorbed denitrifying bacteria. Serving as background, some factors relevant to this work are discussed in the following sections.

The extensive use of nitrogen fertilizers in agricultural areas may create a pollution problem in terms of nitrate contaminated surface waters and ground water supplies (Nilsson 1980). This accelerating contamination could be handled in several ways. The reasons for these problems must be reduced but, in the foreseeable future one will also have to deal with the symptoms of the excessive use of nitrogen fertilizers. This has in recent years initiated a search for efficient nitrate reduction procedures applicable to pure water systems.

In order to reduce the contamination and eutrophication of receiving waters, much work has been done to lower nitrogen concentrations in organic and high-nitrate waste water. There are several excellent reviews covering the field of microbial waste water denitrification (e.g. Christensen and Harremoes 1972; Focht and Chang 1975; Francis and Callahan 1975; Christensen and Harremoes 1977; Schroeder 1980; Prakasam and Krup 1980; Krup and Prakasam 1981; Prakasam and Krup 1982). These denitrification methods can primarily be characterized by the way in which the bacteria are retained within the system. In suspended sludge processes the cell mass is kept within the system by gravity separation and sludge recycling. In attached growth systems the bacteria are retained by adhesion to inert media and the waste water is brought into contact with the attached biofilm. There are several ways of operating an attached growth process; using up-flow and down-flow filters, rotating disc reactors etc. The down flow filters with large inert particles and low flow rates are often termed packed bed reactors. The up flow filters are characterized according to the critical flow, which is the flow required to fluidize the filter media. The rotating disc process is another approach where the inert media is moved through the water. Though microbial denitrification

is not commonly applied as a tertiary step in waste water treatment plants, the technology is well documented and there are several full scale applications throughout the world. The processes developed for pure water denitrification are to a certain extent based on procedures for waste water treatment.

Effects of high nitrate concentrations

As previously stated industrial waste waters, effluents from septic tanks and the wide use of fertilizers may result in high concentrations of nitrate in potable water supplies. The presence of high nitrate concentrations in pure waters poses several problems, e.g., bacterial growth in potable water distribution networks, methaemoglobinemia in infants and formation of potentially carcinogenic nitrosamines. It is not known that nitrate itself is toxic but the reduction of nitrate to nitrite in the gastrointestinal tract, the resorption of nitrite in the small intestine and the subsequent oxidation of haemoglobin to methaemoglobin can cause cyanosis, methaemoglobinemia in infants under six months of age. This is due to the limited oxygen transfer capacity of methaemoglobin.

It has been established that nitrate together with secondary or tertiary amines, can form different nitrosamines under conditions prevailing in the stomach (Magee 1971). It should be mentioned in connection with these problems that the European Community has adopted a standard permitting maximum $50 \text{ mg NO}_3^-/\text{l}$ ($11.3 \text{ mg NO}_3^- \text{-N/l}$) in drinking water (Richard et al, 1980). It could also be of some importance to note that waters with nitrite concentrations exceeding 0.02 mg/l are considered unsuitable for human consumption (Sweden).

Methods for nitrate reduction

In addition to microbial denitrification there are several nitrate removal methods available. Reverse osmosis, ion exchange, chemical denitrification and electrodialysis can be mentioned. These methods have been compared and evaluated by e.g. Sutton et al (1975); Gauntlett and Craft (1979); Adam

(1980); Sorg (1980); Gregory and Sheiham (1981). It has been concluded that microbial denitrification and ion exchange are, for economical reasons, the two methods of choice in pure water processes. Ion exchange is considered more suitable for underground water relatively free of dissolved organic material. Biological denitrification however, is more applicable to surface waters.

Denitrification

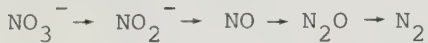
Generally, microbial denitrification refers to the anaerobic, dissimilative reduction of nitrate or nitrite to gaseous products by facultative bacteria. Denitrification is highly interesting not only because of its potential in water and waste water treatment but it is also a major reason for the depletion of nitrogen fertilizer in agriculture. Denitrification is also important as it contributes to the increasing content of N_2O in the atmosphere, which in turn is involved in atmospheric reactions causing deleterious depletion of ozone (Delwiche and Bryan 1976).

Relatively few species of bacteria are able to denitrify and these are found in about 30 genera (Ingraham 1980). The ability to denitrify is relatively uncommon but still found in almost every major physiological and taxonomic group of procaryotes. It should be noted that many facultative anaerobes are capable of nitrate respiration, i.e. they utilize nitrate as the terminal electron acceptor but are unable to further reduce the nitrite formed. It is debatable if all statements on the "reduction of nitrate" in e.g., Bergey's Manual of Determinative Bacteriology (1974) refer to production of nitrite or gaseous products (Ingraham 1980).

One interesting, but difficult to answer, question is which denitrifying bacteria are quantitatively the most significant in different environments. The enumeration techniques seem to favour pseudomonads and other gram-negative rods and the genus Pseudomonas is the most commonly isolated denitrifying bacteria in soil (Ingraham 1980). The available evidence

also suggests that Alcaligenes spp are numerically important in soils (Knowles 1982). In waste water treatment, where methanol is often added as carbon source, Hyphomicrobium spp appear to be dominant. It has been stated (Nurse 1980) that Hyphomicrobium spp would be the only bacteria able to denitrify with methanol as the carbon source, thus explaining the high numbers of this genus in the denitrifying sludges.

Though it is well established that the initial step in denitrification involves the reduction of nitrate to nitrite there have been many conflicting reports in research literature concerning the intermediates between nitrite and nitrogen gas. However, at present the following pathway has been established:



The role of nitric oxide (NO) and nitrous oxide (N₂O) is still under debate and there are different models proposed as to whether nitric oxide is an obligatory intermediate of the process or not (Payne 1980).

Factors controlling denitrification: It is difficult to state physiological properties which are characteristic for all denitrifying organisms. However, these organisms do share the preference to oxygen and they have similar growth requirements. The denitrification process is also influenced by parameters like pH and temperature.

As early as at the end of the 19th century the first study of oxygen effects on denitrification was performed. Since then there have been many contradictory results on the sensitivity towards oxygen. The reports claiming denitrification under aerobic conditions were probably in error due to experimental difficulties (Bryan 1980). Rapidly respiring organisms tend to deplete oxygen in the micro-environment. It is clear that oxygen affects activity as well as the synthesis of the different denitrifying enzymes (Payne 1973, Knowles 1982).

Denitrifying organisms can utilize a wide variety of organic acids, carbohydrates and other organic compounds as carbon source. When applying denitrification on a larger scale in e.g. waste water treatment numerous carbon sources have been utilized (Christensen and Harremoes 1977). It must be emphasized that in waste water denitrification methanol and internal (endogenous) carbon sources are dominant. The quantitative requirements of methanol have been described e.g. by McCarty et al. (1969). The carbon sources employed in the rather few applications concerning drinking water are described in the following section.

Various investigations have shown that copper and iron are required for denitrification (Bryan 1980) as well as sulphur, magnesium and molybdenum. Molybdenum is, e.g., an integral part of many nitrate reductases studied and is necessary for the enzyme activity. Pure culture studies with Pseudomonas denitrificans revealed that the denitrification rate was maximal at pH = 7.0 and 50% of maximum at pH = 6.0 and 9.0 (Dawson and Murphy 1972). Essentially the same results were obtained in suspended growth waste water denitrification by Davies and Pretorius (1975). However, it should be kept in mind that denitrification itself produces alkalinity which means an increase in pH. As shown by Arvin and Kristensen (1982) the pH-value within a denitrifying biofilm was considerably higher than in the bulk liquid. Having dense biofilm differences of two pH-units can be experienced. This is important as the pH, to some extent, influences the product formation.

As for temperature dependence, it is generally established that a temperature of about 30°C is optimal for the denitrification process. However, the occurrence of both thermophilic and psychrophilic denitrifying bacteria is reported. In this context it is probably important to distinguish between psychrophilic growth and psychrophilic denitrification. It was shown by Bergman (1983) that Pseudomonas spp capable of growth at +4°C did not exhibit a higher denitrifying capacity at +11°C than did a mesophilic Pseudomonas denitrificans. When applying denitrification to waste water treatment it has

been shown that a sludge of psychrophilic characteristics is advantageous and can denitrify well down to 2°C (Halmö and Eimhjellen 1981).

Microbial denitrification in potable water treatment

The procedures developed in waste water denitrification have also been employed in attempts to reduce high nitrate concentrations in potable waters. One important difference distinguishing between these processes is that pure water denitrification always requires the addition of a carbon source to the water. The concentration of organic matter in waste water is often sufficient to maintain the reduction process. If addition is required, methanol is the preferred choice in waste water treatment but in pure waters, less toxic carbon sources are desirable.

One of the first pure water denitrification processes described in the literature (Klotter 1969) fully adopted waste water treatment procedures. Ground-water supplemented with skim-milk powder or saccharose was introduced to an aerated basin where an active sludge developed. The sludge-containing water was transferred to an anaerobic reactor where the actual denitrification was performed. After clarification and sludge recycling the process was complete. More recent attempts have mostly employed surface attached biofilms supporting heterotrophic or autotrophic denitrifying bacteria. A few examples of pure water denitrification processes are given in Table 3. It is often very difficult to compare denitrification rates and treatment costs in different investigations, but attempts have been made by Gauntlett and Craft (1979). Examinations of treatment costs have indicated that fluidized bed denitrification is, in most scales of application, the most economical alternative. On the smallest scale however, the costs are similar to those of other possible reduction methods. Roennefahrt (1983) indicated a total cost of 0.95 DM/m³ water treated in a 6,000 m³ per day plant. It should be noted that no, or very few, processes have been tested in full scale operation.

Table 3. Examples of pure water denitrification processes.

<u>Type of process</u>	<u>electron donor</u>	<u>reference</u>
suspended biomass, mixed culture	skim-milk powder, saccharose	Klotter (1969)
suspended and attached biomass, mixed culture	methanol	Gauntlett and Craft (1979)
attached biomass, mixed culture	hydrogen	Ginocchio (1980)
attached biomass, mixed culture	ethanol, acetic acid	Richard et al. (1980)
fibre encapsulated biomass, pure culture	ethanol	Mattiasson et al. (1981)
attached biomass, mixed culture	methanol	Dunning (1981)
attached biomass, mixed culture	methanol	Gauntlett and Zabel (1982)
attached biomass, mixed culture	ethanol	Roennefahrt (1982)
attached biomass, mixed culture	ethanol	Philipot et al. (1983)
attached biomass, mixed culture	sulphur	Philipot et al. (1983)

E SUMMARY AND DISCUSSION OF RESEARCH PAPERSSTUDIES ON ALGINATE-ENTRAPPED PSEUDOMONAS DENITRIFICANS CELLS
IN MICROBIAL DENITRIFICATION (Papers I and II)

Microbial denitrification has, in recent years, been suggested as a method of reducing high nitrate concentrations in contaminated potable waters. In these reactors, suspended or surface attached denitrifying mixed cultures have been utilized.

As entrapped microorganisms have been used in various applications it appeared attractive to employ gel-entrapped, respiring bacteria in this type of reduction process. Alginate immobilization was chosen as it is considered a gentle procedure. This was confirmed in our study as 80 to 90 % of the entrapped viable cells could be recovered on gel solubilization. In denitrification it is important that viable cells are at hand and attempts to immobilize the Pseudomonas denitrificans cells using more brutal techniques, e.g. entrapment in polyacrylamide gel, resulted in the accumulation of nitrite in the effluent which in turn indicated a loss in viability (Nilsson and Ohlson 1981).

Since pure water lacks sufficient amounts of organic material for complete nitrate reduction, an addition of carbon source is required. Ethanol and potassium aspartate were selected as suitable carbon sources. Methanol, the most widely used carbon source, could not be utilized by Pseudomonas denitrificans. This was hardly surprising as it has been indicated that Hyphomicrobium spp are the only organisms accomplishing these reactions (Nurse 1980). Using both suspended and immobilized P. denitrificans cells, we found that the addition of carbon source must give a carbon to nitrate-nitrogen ratio of 1.5 - 2.0 in the water in order to achieve a complete nitrate reduction. This value was applicable for ethanol as well as for aspartate and can be compared with methanol requirements reported as being in the range 1.0 to 1.5 (McCarty et al. 1969).

As expected, the gel entrapped microorganisms suffered from

diffusional limitations, indicated by, e.g. the rise in apparent K_s -values on immobilization. The immobilized cells exhibited an apparent K_s -value of 13.6 mg NO_3^- -N/l compared with 0.3 mg NO_3^- -N/l for the suspended cells. K_s -values reported in the literature for suspended and attached growth reactors (0.1 - 1.0 mg NO_3^- -N/l, Gauntlett and Craft (1979), also confirm that our alginate beads ($\varnothing$ 2 mm) are restricted to diffusional limitations. Reduction of the particle size from 2 to 0.5 mm did improve the situation (Paper II) and even smaller particles can be produced (Klein et al. 1983; Nilsson et al. 1983). However this may increase the leakage of cells, which in turn is a drawback if potable waters are to be treated. In our experiments the cell concentration in the effluent was approximately 10^5 to 10^6 cfu/ml. To minimize this leakage attempts were made to stabilize the gel using cross-linking agents such as glutaraldehyde and polyethyleneimine. Such methods have proved efficient on yeast preparations (Birnbaum et al. 1981) but resulted in a high loss of activity of the Pseudomonas denitrificans cells. These differences may well be due to the different cell wall composition in yeast and bacteria.

Another crucial factor in these systems is the gas production. As nitrogen is sparsely soluble in water, gas bubbles will accumulate in the gel matrix. The problems of excess gas production in agar and Ca-alginate have been discussed by Krouwel and Kossen (1980 and 1981). In our investigations excess gas production caused a very characteristic rupture of the gel beads, identical to that described by Krouwel (1982). This bead rupture probably also contributes to the cell-leakage into the surrounding suspension. It is possible that the use of smaller particles with a shorter diffusion distance could to some extent circumvent the problems of rupture.

Other factors of importance are the possibilities of regenerating lost reduction capacity and the occurrence of side reactions. Our gel preparations exhibited a half-life of 1 - 2 months. This means that if the preparations are to be used under a prolonged period of time there is a need for the regeneration of lost activity. This can be done by the administration of nutrient media to the reactor. The restoration

of initial activity by nutrient addition was entirely due to cell proliferation within the gel. Similar results for Arthrobacter simplex were shown by Ohlson et al. (1978). However, the mechanisms behind the increased activities reported, caused by nutrient dosage have been discussed much. There has also been a debate concerning the importance of side reactions and by-products when employing immobilized viable cells. This is particularly important in processes where the product is to be used for human consumption. In denitrification of water, substances like nitrosamines have to be considered. Philipot et al. (1983) examined denitrified water in the search for different nitrosamines, but none were detected.

The temperature dependence of the nitrate reduction process is important, as the temperature of most water supplies and groundwaters is 5 to 10°C. These low temperatures were not investigated in this study, but it was shown by Bergman (1983) that the mesophilic Pseudomonas denitrificans denitrified at the same rate at 11°C as did psychrotrophic Pseudomonas spp. The long-term low temperature adaptation described by Christensen and Harremoës (1977) was however not investigated.

The rather tedious and elaborate production of cell mass and immobilization procedures may from an economical point of view hamper the use of entrapped microorganisms in microbial denitrification. Another drawback concerning alginate gels in particular, is the need for calcium ions to maintain the gel structure. A water of medium hardness (14 °dH) contains however enough calcium to do so.

As shown in Papers I and II there is always an intermediate production of nitrite in the denitrification process and as very low concentrations are accepted in potable water (Sweden: 0.02 mg NO₂⁻/l) this to some extent governs the dilution rates possible. Due to the diffusional limitations it is obviously difficult to employ entrapped cells in reduction processes where the substrate has to be completely eliminated. This requires very low flow rates.

To reduce certain problems concerning gel entrapment, e.g., cell leakage, other immobilization techniques may be preferred. Encapsulation in a hollow-fibre reactor would theoretically eliminate the leakage of cells and such reactors have been tried (Mattiasson et al. 1981).

In summary, alginate immobilization proved a gentle immobilization technique, giving viable preparations capable of microbial denitrification. However, possible advantages like increased cell density and limited cell leakage were not very clear. As used in this study, the alginate gels exhibited diffusional limitations not acceptable. Producing a low-price product, the techniques used in this study for biomass production, harvesting and immobilization appears too expensive. An alternative could be to grow the cells within the gel matrix but if entrapped cells are to be used in potable water treatment, other matrices and procedures should be used to obviate the disadvantages shown by alginate gel.

It may be symptomatic of these applications that the pure water denitrification processes recently developed in other countries mostly employ surface attached microorganisms. As shown in the following section immobilization by means of attached biofilms is in many ways advantageous compared to the "conventional" entrapment techniques.

STUDIES ON ATTACHED MICROBIAL FILMS IN CONTINUOUS CULTURE (Papers III - VIII)

The phenomenon of cell adsorption onto solid surfaces can be advantageously used in continuous reactors. Depending on the process, the attached organisms are used under growth or non-growth conditions. It is important to distinguish between two attachment procedures. One is to adsorb non-growing organisms to an inert surface and then use this biomass for some kind of bioconversion. Another is to promote adsorption of growing cells and the development of a macroscopic biofilm in a continuous culture. This attached biomass can then be used in the presence or absence of growth media. The term "adhesive fermentation" was proposed in 1976 by Engelbart and Dellweg to

differentiate fermentation processes employing attached micro-organisms from those using organisms dispersed in the medium. If the reactor is used under growth conditions, cells are continuously released from the biofilm. In such a process, both attached and suspended cells contribute to the total productivity. The distribution between adsorbed and suspended cells is of course dependent on the dilution rates and substrates applied. As previously described many factors influence the initial adhesion to a solid surface. These initial events are essential but the biofilm build-up rate is of equal importance and there are several ways to enhance biofilm formation.

Pseudomonas putida ATCC 11172, chosen as the test organism in these experiments (Papers III to VII), has a pronounced ability to form biofilms. Wall growth was exhibited in continuous culture at various dilution rates. A drastic increase in biofilm build-up was observed when dilution rates above the $\mu_{\max}$ of the cells ($\mu_{\max} = 0.6 \text{ h}^{-1}$ in batch culture) were used (III). It appears that the application of dilution rates above those which normally causes wash-out could be one way to enhance biofilm formation. Of course these changes in flow rate have to be made with care and knowledge of the growth characteristics of the organism in question. It has previously been reported that environmental factors influence the initial adhesion of microorganisms to surfaces. No significant effects on the biofilm formation rates could be observed in this study (Paper IV) when the cell concentration, dissolved oxygen tension or temperature was altered. The size of the individual cells was, however, influenced by the temperature and dilution rate. Effects on individual cell size have also been described by Wardell et al (1980), growing an Edwarsiella sp in continuous culture. Considering pH effects, we observed (Paper IV) an increase in the biofilm build-up rate when the pH-value was increased from 5.5 to 6.7.

An increase in dilution rate of the continuous culture to values above the $\mu_{\max}$ of the cells drastically changed not only the biofilm formation rate but also the hydrophobicity

and flocculation capacity of the cells (Paper V). As indicated by, e.g., Kjelleberg (1980) hydrophobicity is a very important factor in adhesion processes. It is quite clear that several characteristics of cells grown at low dilution rates differ substantially from those of cells derived from biofilms. This is true when considering e.g. individual cell size (Paper IV), initial adhesion capacity of nongrowing cells, hydrophobicity and flocculation capacity (Paper V). However, the mechanisms behind the changes in cell properties demonstrated in our experiments have not been investigated. These changes are often attributed to variations in cell wall composition or production of various exopolysaccharides. Physiological differences between free and immobilized organisms have frequently been demonstrated, but may in some cases be explained by the different conditions prevailing on a surface or in the surrounding bulk liquid.

It appears that manipulation of the dilution rate is an efficient alternative to other methods described (starvation of organisms, pretreatment of surfaces) when trying to enhance adhesion and biofilm formation. There is however a need to further investigate the mechanisms behind the changes in organism characteristics.

It is well known that biofilms have a detrimental effect on evaluations of chemostat cultures. In addition, it is often important to study effects of biofilm formation. Valuable comparisons can be made if investigations are performed with and without biofilm build-up. In Paper VI sand administration into the reactor is suggested as one way to eliminate the attached growth. It is shown that the sand containing cultures of P. putida approximately behave as a conventional chemostat and certain growth characteristics can be estimated. Another approach to delay biofilm build-up, is to silicize the fermentor surfaces (Ratnam et al. 1982). However, this procedure has to be reiterated at intervals. Other means described in previous sections are e.g. the use of various scraping devices. There is one more reason to investigate the administration of inert particles into a stirred reactor.

This may be one means of influencing the distribution between attached and suspended organisms and controlling the biofilm thickness. As shown in Paper V, the age and thickness of a biofilm actively formed in a continuous culture very much influence the specific activity of the attached biomass. It has been described previously that the active film thickness is often less than 0.1 mm.

Attached growth can advantageously be used in the degradation of phenolic waste (Hill and Robinson 1975, Scott and Hancher 1976, Sayama and Itokawa 1980, Takahashi et al. 1981). By flow rate manipulations phenol degrading P. putida biofilms could rapidly be produced (Paper VII). It is concluded that biomass adsorbed onto the inert reactor surfaces considerably increases the productivity. The influence of different surface area to volume ratios on the aerobic degradation of phenol was investigated in a 1 l and 4 l fermentor. The phenol reduction capacity increased threefold when the surface to volume ratio was increased five times (0.5 g phenol/l in the substrate). With an increase in the area available for attached growth (increased number of baffles) the cultures were as expected rather insensitive to drastic changes in the dilution rate. The biofilm build-up rate was well in accordance with the values previously presented for asparagine cultures (Papers III and IV). The steady state biofilm held approximately 2×10^8 cfu/cm² and the total attached biomass was about 1 mg (dry weight)/cm².

When designing a biofilm reactor for an aerobic process requiring high oxygen transfer capacities, it is not only a question of providing surface but of providing surface available for attached growth. Suspended particles per se provide a substantial surface area but the attrition forces do not permit attachment (Paper VI). It is also concluded that the design and area of the inert surface (baffle-arrangement) strongly influence the oxygen transfer capacity of the reactor. This is previously documented in the literature e.g. by Enfors and Ericson (1975). It can be discussed

as to what reactor configurations are optimal in order to achieve a high oxygen transfer capacity supporting a maximal number of attached microorganisms. A schematic representation of the reactor used in this study with increased baffle surfaces is given in Figure 3.

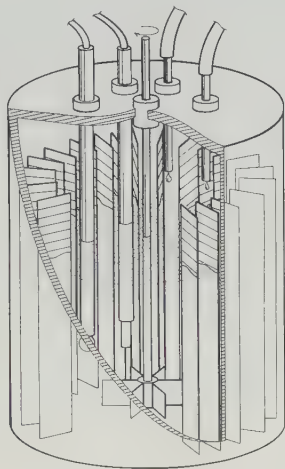


Figure 3. Schematic representation of a reactor with increased baffle surfaces available for attached growth.

In recent years fluidized bed reactors have frequently been proposed as the most adequate reactor configuration using attached biomass. However, in aerobic processes, where intensive mixing is required, the capacity is very dependent on the shape and porosity of the inert carrier. The process stability of these reactors is also influenced by the accumulation of attached biomass, as excess biofilm production can cause sedimentation or flotation of the carrier material. A fixed film reactor optimally designed may well be a useful alternative.

As previously discussed, precoating of surfaces by natural or synthetic polymers is one way to enhance biofilm formation. Experiments performed in our laboratory (Nilsson and Dostalek 1984) show that the pretreatment of glass surfaces with positively charged polyelectrolytes, significantly increased the attachment of non-growing Pseudomonas putida cells. This may

be one way to further increase biofilm build-up rate in the fixed film bioreactor.

As previously concluded, gel entrapment is from certain aspects unsuitable for microbial denitrification. The reasons are e.g. the limited mechanical stability of the gel matrix and the diffusional limitations exhibited. If surface attached biofilms are employed, the "immobilization" procedure is substantially simplified and the costs are thereby reduced.

Gauntlett and Zabel (1982) claim that 10 to 14 days are required in order to attain full denitrifying capacity in a mixed culture fluidized bed reactor with methanol as the carbon source in a nitrate contaminated river water. Our studies with P. denitrificans show that at biofilm will develop more rapidly, particularly under aerobic conditions if a complete growth medium is used and the dilution rate is increased to values above the $\mu_{\max}$ (Paper VIII). The pure culture of P. denitrificans was grown in an aerobic continuous culture on L-asparagine. In accordance with P. putida this organism exhibited attached growth at dilution rates above the $\mu_{\max}$. The biofilm steady state values of 10^8 cfu/cm² and a total biomass of 1 mg (dry weight)/cm² are identical to those of the P. putida culture (Paper VII). After a two hour adaption period the aerobically produced biofilm cells showed a nitrate reduction capacity close to that of cells grown anaerobically in the presence of nitrate. These surface attached resting cells exhibited a stable denitrification capacity for about 30 days when exposed to high nitrate water (100 mg NO₃⁻/l). If the shearing forces applied are moderate, the cell leakage into the effluent in this reactor ($10^6 - 10^7$ cfu/ml) is of a similar magnitude as shown by the alginate gel reactor. However, if the denitrified water is to be infiltrated via, e.g., a sand bed prior to distribution the contaminating organisms may not be as crucial as when the water is to be used for immediate consumption.

Whether using entrapped or surface adsorbed microorganisms it is important to realize that diffusional limitations very

much govern the possible loading rates. Apparent K_s -values in the range 0.1 to 1 mg NO_3^- -N/l for suspended and attached growth reactors are reported in the literature (Gauntlett and Craft 1979). These values are to be compared with 13.6 mg NO_3^- -N/l previously calculated for the alginate entrapped cells (Paper I). As nitrite reduction is often the rate limiting step, accumulation of this intermediate is liable if the flow rates employed are too high. This is a severe problem as the allowed nitrite concentration in potable water is 0.02 mg NO_2^- /l compared to 30 mg NO_3^- /l (Sweden).

As previously described, attempts have been made in several countries to use surface attached microorganisms, pure or mixed cultures, in the denitrification of potable waters. Still, many problems remain for further discussion. Effects of residual carbon-source, removal of microorganisms present in the water, use of efficient dosage and control devices, possibilities of side reactions and byproduct formation and reduction of diffusional limitations in biofilms are a few factors for further evaluation.

F CONCLUDING REMARKS

The use of immobilized whole cells as an alternative to "conventional" fermentation has been suggested in recent years. Immobilization procedures influence in many respects the characteristics and transformation capacities of the microbial cells. Immobilized cell preparations have many merits but also several drawbacks. Some of these were previously discussed and this study, to some extent, confirms that alginate gel entrapment in low-price bulk chemical production or detoxification processes suffers very much from drawbacks like limited mechanical stability, diffusional limitations etc. Entrapment may be a useful alternative in small scale production of expensive products where the advantages of these systems can be fully utilized. However, the demands on the gel matrices in terms of mechanical integrity, diffusion properties, biocompatibility etc require further research in this field prior to industrial application.

One immobilization technique rather neglected is the adsorption of microorganisms onto inert surfaces. This principle is most widely established in nature and has been empirically utilized for many years in , e.g., waste water treatment. The deliberate production of biofilms in a continuously operating reactor, "adhesive fermentation", has been suggested as an advantageous use of immobilized cells. Due to nutrient accumulation on surfaces very dilute substrates can be processed at flow rates giving wash-out in a suspended growth reactor. This immobilization procedure is more simple to perform than entrapment. An important issue in this field is to enhance and control the biofilm formation. Some enhancement procedures have been demonstrated in the literature and flow rate manipulations are suggested in this study as another means "of speeding up" the attachment process. It is clear that dilution rate influences cell characteristics important to attachment and biofilm formation.

When gel entrapped and surface adsorbed cells are compared in a denitrification process it appears that, though the comparisons are made from non-optimal conditions, the attached

biofilms are advantageous. This conclusion is partly based on the simplicity of the attachment procedure and partly on the fact that the merits of the entrapment technique can not be fully utilized in this process. The other model system employed, aerobic degradation of phenol, also proved suitable in a fixed biofilm reactor. To improve this process, work has to be done concerning reactor configurations answering to the high mass transfer requirements.

One area for future biofilm research could be the development of methods generally enhancing attachment also for organisms which normally do not exhibit adhesive properties. Other areas, not considered in this study but very important, are the kinetics of fixed biofilm reactors and the optimal design of such vessels. Several kinetic models concerning wall growth have been presented in recent years and much work has been done to produce carrier material suitable for biofilm production.

Finally it is my opinion that, when estimating the future use of biofilms in various fermentation processes one can travesty the words of Sir Winston Churchill: This is not the end, it is not even the beginning of the end but rather the end of the beginning.

G ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to my adviser, Professor Nils Molin, for introducing me to the field of microbiology, for support and encouragement throughout this work and for providing the most excellent working facilities. I also want to thank Lena Häggström, Sten Ohlson and Klaus Mosbach for most stimulating co-operation in the early, "entrapped", part of the work. For inspiring collaboration and many stimulating discussions I am most grateful to Göran Molin, Milan Dostálek and Lena Stenson-Holst.

During this work I have received invaluable assistance from many people. I particularly wish to thank Maria Wilhelmsson and Birgitta Sörenby for their competent and enthusiastic work, without which I would not have managed. I am also grateful to Karl Mohlin and Jan Sjöholm for solving all electrical and mechanical problems turning up at the most inconvenient times. Kristina Alm and Kajsa Eriksson are gratefully thanked for typing manuscripts not always easily legible. I am thankful to Barbara Goldschmidt, Kristina Dunér and Jan-Olof Bergman who performed excellent undergraduate work. Peter Corrigan is acknowledged for his linguistic advice and Lars Seiron for his kind help with photographic work.

I would like to thank my colleagues Olle Holst, Lena Hansson, Inga-Maj Stenström, Stefan Rydin and Clas Lönner, with whom I occupied the remote part of the department, for all their support and helpful comments during my work. All other friends at the Department of Applied Microbiology, thank you so much for all your help and support.

Finally, I am most grateful to Ruth, my wife, for typing almost illegible manuscripts, for putting up with the rather messy situation during the preparation of this manuscript and for a never failing support.

The financial support given by the Swedish National Board for Technical Development and the Swedish Biotechnology Research Foundation is gratefully acknowledged.

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Denitrification of Water Using Immobilized *Pseudomonas denitrificans* Cells

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Summary. Preparations of living *Pseudomonas denitrificans* cells immobilized in alginate gel were used in the denitrification of water. In the presence of an exogenous carbon source the entrapped microorganisms reduced nitrate and nitrite to gaseous products and to achieve complete reduction, carbon to nitrogen ratios of over two were required. The effects on denitrification of particle size and the number of bacteria in the gel were investigated. Apparent K_m values for nitrate and nitrite reduction were calculated for free and immobilized cells. When the immobilized cells were incubated in nutrient media, an increase in reduction rate was observed and this was shown to be caused by the growth of cells within the gel particles. Immobilized *P. denitrificans* cells retained 75% of their initial nitrate reduction capacity after 21 days of storage at +4°C. The operational stability of the alginate-immobilized cells was studied both in batch and in a column which was operated continuously. A column (45 g of alginate-cell fibers in 80 ml) denitrified a high nitrate drinking water (100 mg NO_3/l) with a rate of 300 ml of nitrate and nitrite free water/day/g of gel. The half life for nitrate reduction was estimated to be 30 days.

Introduction

The removal of nitrogenous compounds from various types of water has received widespread attention in recent years (Shuval and Gruener 1975). The possible deterioration of groundwater supplies by the extensive use of nitrogen fertilizers emphasizes the need for efficient processes for the purification of high nitrate waters. Several methods have been suggested for this treatment, including algae ponds, bacterial assimilation, microbial denitrification, ion-exchange, reverse osmosis and chemical denitrification. These various methods have been compared and evaluated (Reeves 1972; Sutton et al. 1975).

It occurred to us that the use of nitrogen reducing microorganisms in their immobilized state could be a useful alternative technique. Apart from earlier isolated studies

(Mosbach and Mosbach 1966; Updike et al. 1969) on immobilized microbial cells, it is only recently that the industrial use of such immobilized cell systems has become well established notably by the work of Chibata and coworkers (for reviews see Abbott 1977; Brodelius 1978; Chibata and Tosa 1977; Durand and Navarro 1978; Jack and Zajic 1977; Klein and Wagner 1978).

In this report we describe the purification of high nitrate drinking water with immobilized (entrapped) *P. denitrificans* cells. Immobilization in calcium alginate gel was selected because it is nontoxic as well as a mild (high recovery of cell activity) and simple method.

Materials and Methods

Chemicals

Sodium alginate was obtained from Sigma Chemical Co., USA. All other reagents were of analytical grade and obtained from commercial sources. Deionized water was used in all procedures.

Culture of Microorganisms

Pseudomonas denitrificans (ATCC 13867) was maintained on agar slants of the following composition (in g/l): glucose 10; yeast extract 5; KNO_3 0.5; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.006; agar 15. The culture was re-streaked every four weeks. The cells used for nitrate- and nitrite conversion were cultivated in the following growth medium (in g/l): potassium aspartate 15; yeast extract 7; peptone 7; KNO_3 8; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 0.0025; MnCl_2 0.0025; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.006; pH 6.75.

The microorganisms were transferred from agar slants to 100 ml of medium of the above composition and incubated for 24 h at 30°C without shaking. The cells were re-inoculated in 1 l of fresh medium (in Erlenmeyer flasks) and grown without shaking for 20 h at 30°C; these were then harvested by centrifugation (10 min, 12,000 g) and washed twice in 0.05 M Tris-HCl, pH 7.5. The cell yield was on the average 9 g w wt./l, corresponding to 1.2 g dry wt./l.

Immobilization in Calcium Alginate

Unless otherwise stated, immobilization was performed as follows: 1 g of centrifuged *P. denitrificans* cells, containing approximately 4×10^{11} living cells/g (wet wt.), was mixed thoroughly with 9 g of a 2% (w/v) sodium alginate solution. The mixture was pumped continuously through a hypodermic needle (0.1 - 1 mm I.D.) and gelled immediately to form beads or fibers on contact with a solution of 0.1 M CaCl_2 . These gel particles or gel fibers were cured in the same solution at room temperature (20° - 22°C) for about 2 h. After washing briefly with water the cell-alginate particles were stored at +4°C before use. About 5 g (wet wt.) of immobilized particles were obtained.

Solubilization of Alginate Gel

Gel particles containing *P. denitrificans* (1 g, average bead size 2.5 mm) were disintegrated by forcing them through a stainless steel filter. The crushed material was suspended in 30 ml of 0.1 M sodium phosphate buffer, pH 7.5, and shaken at 21°C for 30 min, after which solubilization was complete. Depending on further use, the suspension was either centrifuged (10 min, 12,000 g) or further diluted.

Determination of Nitrate and Nitrite

Nitrate concentrations were determined using a nitrate electrode (model 93-07, Orion Research, USA). The potential developed was measured against a double junction reference electrode. Nitrite concentrations were measured colorimetrically according to Standard Methods for the Examination of Water and Waste Water (1975).

Assay of Nitrate Reduction Rate

The cells (typical amounts: free, 0.4 g wet wt.; immobilized, 2 g wet wt.) were suspended in 40 ml of non-growth medium containing ammonium sulphate (0.21 g), KNO_3 and potassium aspartate; the organic carbon (as aspartate) to nitrogen (as nitrate) ratio (i.e., C/N) in the assay was 6. Nitrogen gas was bubbled through the suspension and the nitrate reduction was initiated by adding the substrate KNO_3 ; the concentration of nitrate—nitrogen (i.e., mg NO_3^- -N/l) used in routine assay was usually in the range of 0.5 to 25 mg. The progress of the reaction was recorded continuously at 21°C using the nitrate electrode. Nitrate reduction rate was determined as the consumption of mg of NO_3^- -N/min/g (wet wt.) for free cells and mg of NO_3^- -N/min/g gel (wet wt.) for immobilized cells.

Assay of Nitrite Reduction Rate

Nitrite reduction was studied colorimetrically using NaNO_2 as the substrate; 0.2 - 25 mg nitrite-nitrogen/l (i.e. mg NO_2^- -N/l). The assay was carried out as above, except that ammonium sulphate was not used and the nitrogen in the C/N ratio refers to NO_2^- -N. The nitrite concentration was determined in small samples (20 - 200 μl) that were withdrawn at intervals and the nitrite reduction rate was defined as the consumption of mg of NO_2^- -N/min/g (wet wt.) for free cells and of mg of NO_2^- -N/min/g gel (wet wt.) for immobilized cells.

Results and Discussion

The Effect of Different Carbon Sources on Denitrification of Water

Microbial denitrification is an anaerobic process and the oxidation of organic carbon is coupled with the reduction of nitrate, via nitrite, to gaseous compounds, mainly

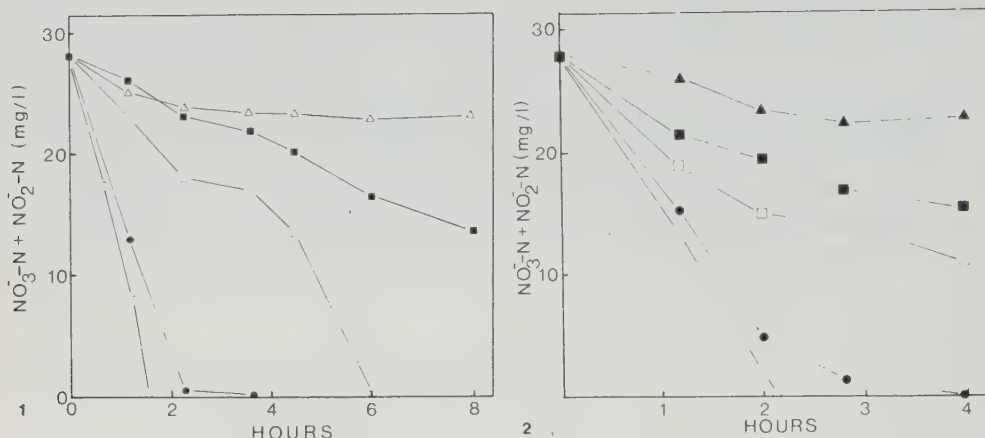


Fig. 1. The effect of various organic carbon sources on denitrification with *Pseudomonas denitrificans* cells. Free cells (0.7 g wet wt.) were incubated under nitrogen atmosphere at 30°C in 200 ml of water, supplemented with 28 mg $\text{NO}_3^- \text{N/l}$ (KNO_3). C/N ratio = 6.0.

(○) potassium aspartate, (●) ethanol, (□) glucose, (■) sodium citrate, (△) no carbon source added

Fig. 2. The effect of different C/N ratios on denitrification with *P. denitrificans* cells. Free cells (0.5 g wet wt.) were incubated under nitrogen atmosphere at 30°C in 200 ml of water containing 28 mg $\text{NO}_3^- \text{N/l}$ (KNO_3) and potassium aspartate.

(▲) C/N = 0, (■) C/N = 0.5, (□) C/N = 1, (●) C/N = 2, (○) C/N = 6

nitrogen (Delwiche and Bryan 1976; Painter 1970; Payne 1973). Denitrification is greatly influenced by the proper selection of the organic carbon source. A wide variety of organic carbon sources has been used for waste water denitrification. (Christensen and Harremoës 1977). The substrate most frequently used is methanol but we did not find it suitable in groundwater purification, mainly because of its toxicity. Figure 1 shows the results obtained with four different carbon sources and indicates a different response of the free, non-growing *P. denitrificans* cells to the different substrates. The initial reduction of nitrate in the absence of a carbon source is caused by endogenous respiration. Considering these reduction rates, we used potassium aspartate as a suitable carbon source in our studies with immobilized *P. denitrificans* cells. Differences in denitrification rates caused by various types of organic substrates have been shown previously (Crabtree 1972; McCarty et al. 1969).

The Effect of Different C/N Ratios on Denitrification

To achieve complete reduction of nitrate and nitrite there is a need for a certain supply of organic carbon, i.e., that the C/N ratio should exceed a certain value. Most of the previous reports on studies of carbon sources and C/N-ratios have focussed on methanol as the carbon source in the treatment of high nitrate waste water. McCarty et al. (1969) proposed the following stoichiometric formula for the total concentration of methanol required for complete microbial denitrification: $C_m = 2.47 (\text{NO}_3^- \text{N}) + 1.53 (\text{NO}_2^- \text{N}) + 0.87 (\text{DO})$, where C_m , $(\text{NO}_3^- \text{N})$, $(\text{NO}_2^- \text{N})$ and DO are the concentrations in mg/l of methanol, $\text{NO}_3^- \text{N}$, $\text{NO}_2^- \text{N}$ and initial dissolved oxygen

respectively. Mixed culture systems also show a similar pattern for methanol requirement (Narkis et al. 1979).

The relationship between the denitrification and C/N ratios for *P. denitrificans* cells is shown in Fig. 2. The lowest C/N ratio required for complete reduction (within a practical time scale) is 2, and in the range from 4 to 6, the reduction rate is virtually the same (C/N ratio of 6 is shown in Fig. 2). From these findings we conclude that the use of a C/N ratio of 6 would ensure complete reduction at a high reduction rate.

Immobilization of Cells in Calcium Alginate

Immobilization of whole cells in calcium-alginate gel has become popular in recent years, primarily because it is a mild and inexpensive method and it is easy to perform (Cheetham 1979; Dallyn et al. 1977; Hackel et al. 1975; Kierstan and Bucke 1977; Ohlson et al. 1979). Furthermore, it is suitable for automatic production of gel particles, for instance 250 g of gel particles could be produced in 1 h by pumping a cell-alginate suspension through a hypodermic needle into a calcium chloride solution.

The found nitrate- and nitrite reduction activities of the immobilized cells depend mainly on two factors. First, survival of bacteria during the immobilization procedure, and secondly the diffusional restrictions on the transport of substrates and products within the polymer matrix.

The viability of entrapped *P. denitrificans* in calcium-alginate gel was estimated by dissolving the gel in phosphate buffer and the number of released cells was measured by conventional viable count. A high percentage (80% - 85%) of the *P. denitrificans* cells could be recovered from the dissolved alginate gel, which shows that toxicity hazards to the cells are not significant in the immobilization procedure. The time course for nitrate reduction with free and alginate-immobilized *P. denitrificans* cells (bead size 2.0 -

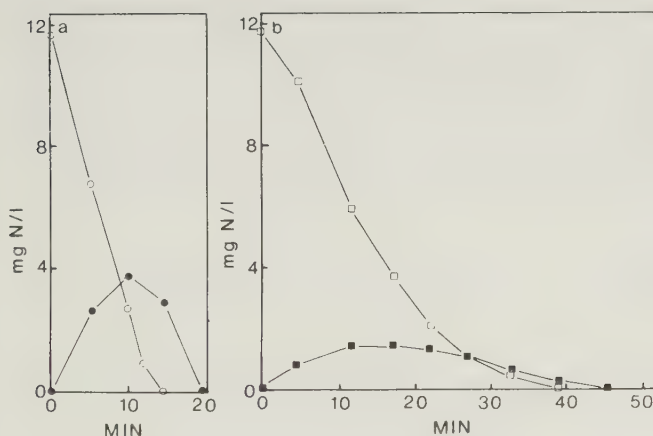


Fig. 3a and b. Time course for nitrate reduction with free and alginate-immobilized *P. denitrificans* cells. *P. denitrificans* gel particles (4 g wet wt., bead size 2 - 2.5 mm) or free cells (0.8 g wet wt. which is equivalent to 4 g of cell-alginate gel), were incubated at 21°C in 80 ml of water containing K-aspartate (375 mg/l) and 12 mg $\text{NO}_3^- \text{N/l}$. Nitrogen gas was bubbled through the suspension. a Free cells, (○) mg $\text{NO}_3^- \text{N/l}$, (●) mg $\text{NO}_2^- \text{N/l}$. b Immobilized cells, (□) mg $\text{NO}_3^- \text{N/l}$, (■) mg $\text{NO}_2^- \text{N/l}$

2.5 mm) is given in Fig. 3, from which it is seen that the immobilized cells have a nitrate reduction rate of about 50% of that of the free cells. In the same figure the intermediate nitrite formation is depicted showing that with some time lag also nitrite is removed. With smaller gel particles (0.5 - 1.0 mm), 70% - 80% of the nitrate reduction rate of free cells was found, again suggesting that alginate immobilization is a mild method. Furthermore, when the size of beads was increased to 3 mm, the nitrate reduction rate declined to 25% of the original activity found with free cells but a further increase to 5 mm diameter did not substantially change the reduction rate. Virtually all (95%) of the original activity could be regained by dissolving these gel beads in phosphate buffer indicating that diffusional limitations were the major cause of the decrease in nitrate-reduction rate when the cells were immobilized in calcium alginate gel.

The effect of diffusional restrictions on nitrate- and nitrite reduction rates is also obvious from Fig. 4, where the cell concentration in the beads (average size of 3 mm) was varied. It is likely here that diffusion is the rate controlling step in nitrate- and nitrite reduction and one way to minimize such adverse diffusion effects is to make very small particles. However, this could cause severe pressure drop problems in packed bed reactors.

One problem experienced with alginate gel is maintaining satisfactory mechanical characteristics of the gel particles during continuous operation. Since multivalent cations, such as Ca^{2+} or Al^{3+} must be present to stabilize the gel, immobilization in alginate gel cannot be used with enzyme systems that are inhibited by these metal ions. It has been reported (Takata et al. 1977) that treatment with hardening reagents, such as glutaraldehyde, stabilized the gel so that metal ions could be omitted. However, this technique might kill the microorganisms, thereby excluding the possibility of activating them with nutrients (see below). This kind of hardening treatment can also give rise to increasing diffusional problems. The abundant production of gaseous products during denitrification with entrapped *P. denitrificans* increased the tendency to rupture the gel

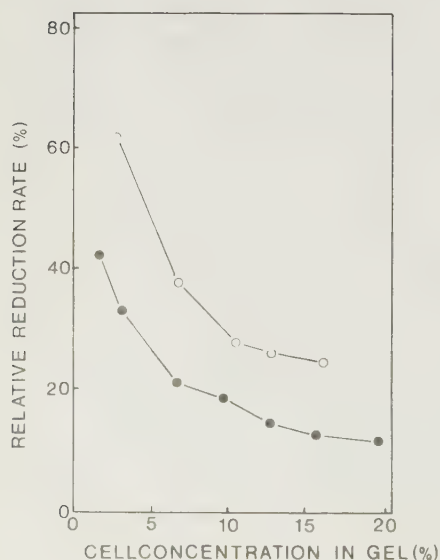


Fig. 4. The effect of cell concentration on the reduction rates of nitrate and nitrite with alginate-immobilized *P. denitrificans* cells. Gel particles (1 g wet wt., bead size 3 mm) were incubated under nitrogen atmosphere at 21°C in 40 ml of medium containing K-aspartate (750 mg/l) and 6 mg NO_3^- -N/l (KNO_3 , nitrate reduction) or 25 mg NO_2^- -N/l (NaNO_2 , nitrite reduction). For each cell concentration the reduction rate of free cells is set at 100%. (●) relative nitrate reduction rate, (○) relative nitrite reduction rate

beads during sustained operation. To guarantee good stabilization, the long-run experiments were carried out in the presence of 5 mM CaCl_2 .

The Effect of pH

The pH activity profiles for both nitrate- and nitrite reduction were examined for free and immobilized *P. denitrificans* cells in the range of 4.5 to 9.0. They were found to be highly similar with the pH-optimum in the range of 6.5 - 7.0. Essentially the same pH-optimum has been reported for other microorganisms in denitrification processes (Davies and Pretorius 1975; Dawson and Murphy 1972).

The Effect of Temperature

The temperature dependence of microbial denitrification is an important parameter for process design in the treatment of high nitrate waters. Nitrate- and nitrite reduction was carried out in the temperature range of 6° to 55°C, and the reduction rates were estimated during a short incubation time (1 h). Our experiments show that there is no shift in temperature optimum for nitrate- and nitrite reduction when cells are immobilized, but the optimum range is slightly broader for the immobilized cells. The optimum temperature was 35°C and above 45°C the nitrate- and nitrite reduction rates declined rapidly both for immobilized and free cells. On the other hand, at lower temperatures, a higher retention of nitrate- and nitrite reduction activities of immobilized cells was achieved; for example, at 18°C about 50% of the maximum nitrate reduction rate remained for immobilized cells compared with 30% for free cells.

Temperature Stability

Improved temperature stability has been reported for many different types of immobilized cells. The stability of immobilized and free cells in terms of nitrate- and nitrite reduction rates was compared at different temperatures (Fig. 5). This indicates that both nitrate- and nitrite reduction activities of the immobilized cells are somewhat more stable than those of the free cells.

*Apparent Michaelis-Menten Constants (K'_m) for Denitrification with Free and Alginate-Immobilized *P. denitrificans* Cells*

The effects of nitrate- and nitrite concentration on the initial reduction rate are shown in Fig. 6a, b. From Lineweaver-Burk plots we could evaluate the K'_m values for nitrogen reduction of free and immobilized cells. The K'_m values for the nitrate reductase system was 0.021 mM (0.3 mg NO_3^- -N/l) for free cells and 0.97 mM (13.6 mg NO_3^- -N/l) for immobilized cells. For the nitrite reductase system the K'_m values were 0.31 mM (4.4 mg NO_2^- -N/l) for free cells and 1.2 mM (17.2 mg NO_2^- -N/l) for immobilized cells.

The significant increase in the K'_m on immobilization can be explained in part to be due to diffusional limitations of substrate transport within the polymer.

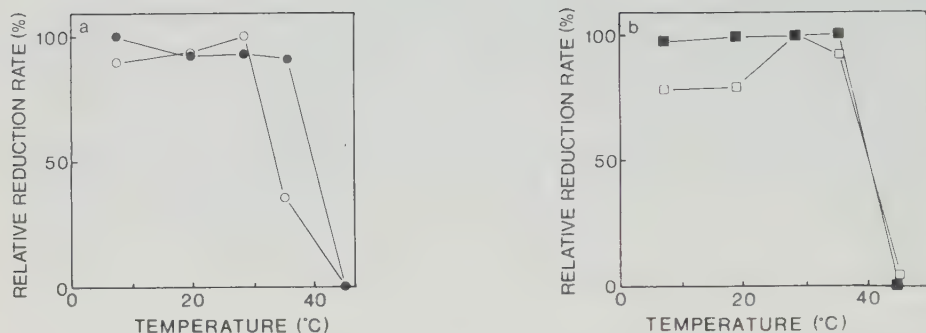


Fig. 5a and b. Temperature stability of nitrate- and nitrite reduction rates of free and alginate-immobilized *P. denitrificans* cells. Alginate-immobilized cells (1 g wet wt.) or free cells (0.2 g wet wt., equivalent to 1 g of alginate gel) were suspended in 40 ml of 0.025 M Tris-HCl, pH 7.5, containing K-aspartate (C/N = 6.0), CaCl_2 (1.1 g/l), and substrate: 660 mg NO_3^- -N/l (KNO_3 , nitrate reduction, or 81 mg NO_2^- -N/l (NaNO_2 , nitrite reduction). The suspension was incubated under nitrogen atmosphere at different temperatures for 20 h (nitrate reduction) or 3 h (nitrite reduction). The residual nitrate- and nitrite reduction rates were determined as described in Materials and Methods. Maximum reduction rate is set at 100%. **a** Nitrate reduction rate, (○) free cells, (●) immobilized cells. **b** Nitrite reduction rate, (□) free cells, (■) immobilized cells

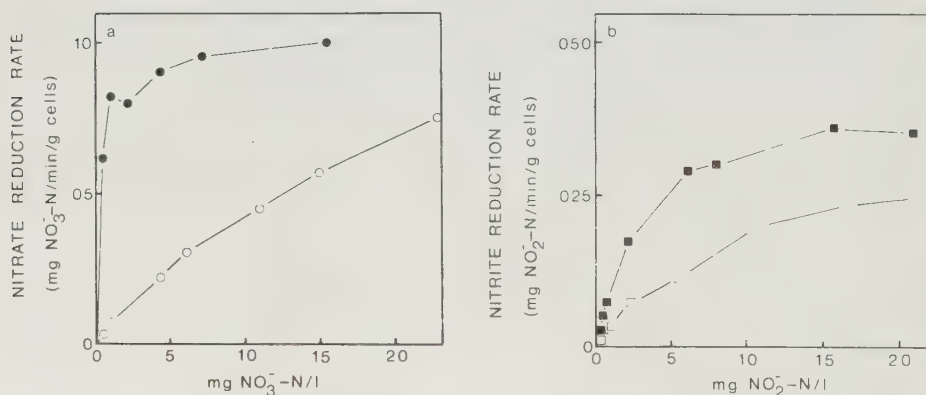


Fig. 6a and b. The effect of nitrate- and nitrite concentrations on initial reduction rates. *P. denitrificans* gel particles (2 g wet wt., bead size 2.5 mm) or free cells (0.4 g wet wt., equivalent to 2 g cell-alginate gel) was incubated at 21°C in 40 ml of water containing K-aspartate (C/N = 6) and NO_3^- -N (KNO_3 , nitrate reduction) or NO_2^- -N (NaNO_2 , nitrite reduction). Initial reduction rates were determined as described in Materials and Methods. **a** Nitrate reduction, (●) free cells, (○) immobilized cells. **b** Nitrite reduction, (■) free cells, (□) immobilized cells.

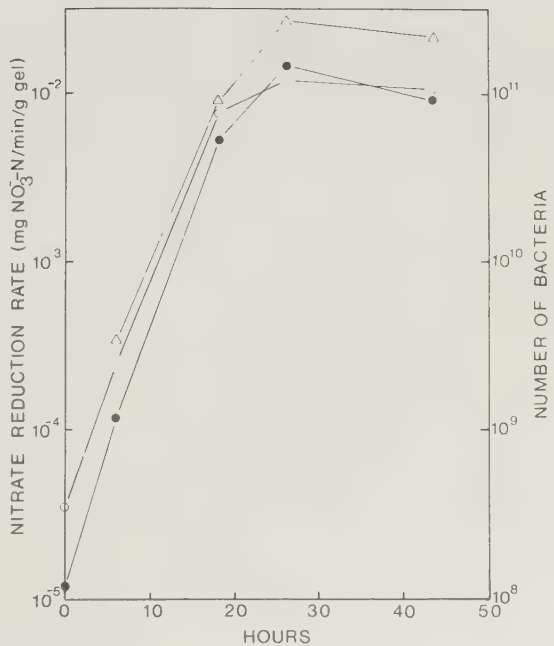
Activation

One of the advantages with the use of viable immobilized cells is the possibility of restoring or even increasing the in situ enzymic activities by nutrient addition (Kennedy et al. 1976; Klein and Wagner 1978; Larsson et al. 1976; Mosbach and Larsson 1970;

Ohlson et al. 1978; Pache 1978). The reasons behind this activation of immobilized living cell systems in nutrient medium have been examined in detail (Chibata 1978; Klein and Wagner 1978; Ohlson et al. 1978, 1979) and from these studies we and other workers have found strong evidence that growth of the bacterial cells is the major cause of activation.

Activation of immobilized *P. denitrificans* cells by nutrient addition, resulting in an increase in nitrate reduction activity, was also observed in this study. The approach we used to study this activation and its possible causes was to dissolve the alginate beads in phosphate buffer and determine the number of immobilized living cells by viable count, as well as their nitrate reduction activity; these results were then compared with the activity expressed for the immobilized cells. Figure 7 shows the results of such a study, which reveals a remarkable increase in nitrate reduction activity of the immobilized cells of up to 300 times their original activity. The relative degree of activation is of course dependent on the initial cell concentration in the gel. The change in the number of living cells resembles to a great extent a growth curve for *P. denitrificans* cells. Moreover, the nitrate reduction activity of free (i.e., released from alginate gel) and immobilized cells closely follows the number of viable cells during the process of activation. However, at higher cell concentrations the enzyme activity of the immobilized cells departed to some extent from that of free cells, most likely because of diffusion resistance to substrate transport in the gel lattice. In conclusion, the results suggest that the activation occurred as a result of cell growth in the polymer matrix.

Fig. 7. The effect of nutrients on the nitrate reduction activity and the number of immobilized *P. denitrificans* cells. Portions (4 g) of *P. denitrificans* gel particles were incubated at 21 °C in 250 ml of growth medium supplemented with CaCl_2 (0.6 g/l). The gel was filtered off at intervals, washed with water and assayed for nitrate reduction activity. After solubilization of the gel beads, the number of viable cells was determined, as well as their nitrate reduction activity. (●) number of living cells/g gel, (Δ) nitrate reduction rate of free cells after solubilization of alginate gel, ($\circ$) nitrate reduction rate of immobilized cells. Logarithmic scales are used



Storage Stability

Storage stability is an important factor in estimating "standby" capacity for immobilized cells. An experiment with *P. denitrificans* gel [initial nitrate reduction rate: 0.0034 mg NO_3^- -N/min/g gel (wet wt.)] was carried out to determine the storage stability in terms of the nitrate reduction activity. After washing the gel in 0.01 M CaCl_2 , it was stored in a moist condition at -18°C and $+4^\circ\text{C}$. Storing the gel at -18°C was not applicable since the particles rapidly lost their mechanical strength. In contrast, at $+4^\circ\text{C}$ the *P. denitrificans* gel retained 75% of its initial activity after storage for 21 days, and the gel particles still maintained their mechanical stability.

Operational Stability

For successful application of an immobilized cell system, it is essential to investigate the stability during long-term continuous operation. At the start of our studies on operational stability, a repeated batch-wise reduction of nitrate and nitrite by alginate immobilized *P. denitrificans* was carried out (Fig. 8a, b). The nitrate- and nitrite reduction rates of the immobilized cells in buffered medium (0.05 M Tris-HCl, pH 7.2) were initially found to be 0.030 mg NO_3^- -N/min/g gel (wet wt.) and 0.047 mg NO_2^- -N/min/g gel (wet wt.), respectively. On the other hand, when the gel was incubated in unbuffered water medium, the reduction rates (both nitrate and nitrite) were considerably lower,

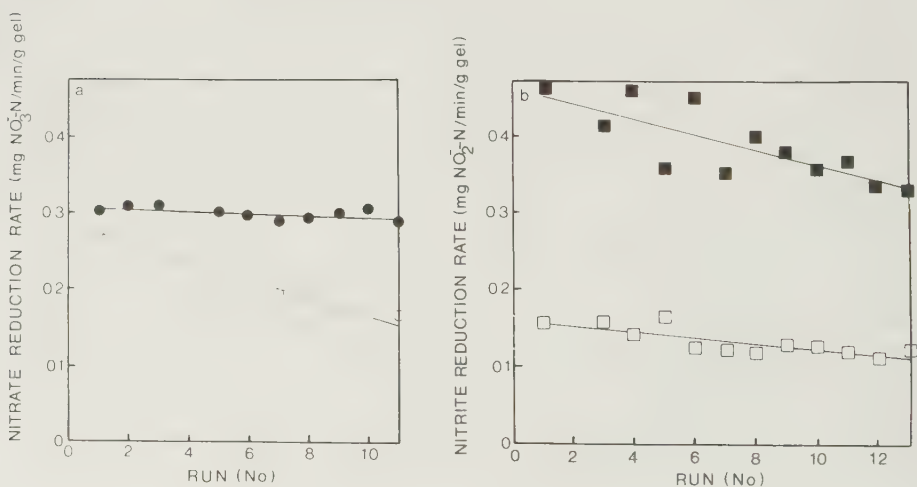


Fig. 8a and b. Repeated, batch-wise reduction of nitrate and nitrite in different media by alginate-immobilized *P. denitrificans* cells. The gel particles [1 g wet wt., equivalent to 0.2 g free cells (wet wt.)] were incubated on a rotary shaker under nitrogen atmosphere at 21°C in 200 ml of medium containing CaCl_2 (1.1 g/l), K-aspartate (C/N = 6.0) and 140 mg NO_3^- -N/l (KNO_3 , nitrate reduction) or 150 mg NO_2^- -N/l (NaNO_2 , nitrite reduction). After each run (12 h), the gel was filtered, washed with water and again incubated with fresh medium. The reduction rates were determined by measuring the nitrate- and nitrite concentrations before and after each run. **a** Nitrate reduction; (●) 0.05 M Tris-HCl pH 7.2, (○) water, initial pH in each run 6.2. **b** Nitrite reduction; (■) 0.05 M Tris-HCl pH 7.2, (□) water, initial pH in each run 6.2

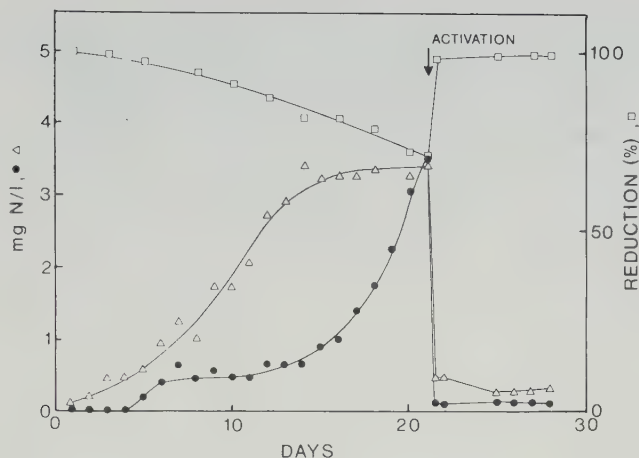


Fig. 9. Continuous nitrate reduction by an alginate-immobilized *P. denitrificans* cell column. Gel fibers (45 g (wet wt.), diameter 1 mm, cell concentration is 8%) were packed in a column (1.6 x 40 cm, total volume 80 ml). The substrate solution, renewed every 48 h and containing CaCl_2 (0.55 g/l), K-aspartate (0.5 g/l) and NO_3^- -N (22 mg/l) was passed at 21°C upward through the column at a flow-rate of 0.54 l/h. At the time indicated, the gel was activated under batch conditions at 21°C in growth medium (see Materials and Methods) for 8 h. (●) mg NO_3^- -N/l in effluent, (Δ) mg NO_2^- -N/l in effluent, (□) reduction of nitrate to gaseous products (% of inlet concentration of nitrate)

probably due to a pH shift to the alkaline side (pH increased from 6.2 to 8.5) during each run. This finding is in agreement with our previous investigation of the pH-effect on nitrate- and nitrite reduction activities of the alginate-immobilized cells.

The nitrate reduction activity in Tris-buffer was almost constant during 5 days (half-life ($t_{1/2}$) = 50 days), which is in contrast to the 'decay' in water ($t_{1/2}$ = 6 days). Another situation occurred with nitrite reduction, where the half-life in both Tris-buffer and water was 11 days. It should be pointed out that the half-life measurements are obtained by extrapolation from a limited amount of data, but the trend expressed in the 'half-life' figures should be essentially correct.

Since our experiments with batch-wise nitrate reduction with *P. denitrificans* gel showed promising results, we used this type of gel for continuous nitrate reduction of high nitrate drinking water. Figure 9 presents the results on operational stability of the *P. denitrificans* gel column. To the high nitrate drinking water (22 mg NO_3^- -N/l) used as a substrate, potassium aspartate was added as the carbon source and CaCl_2 to maintain the gel structure. At the start, the column could produce 0.3 l of nitrate and nitrite free water/day/g gel (wet wt.) and to determine operational stability the flow was adjusted to have a detectable amount of nitrite in the effluent.

The half-life for nitrate reduction to gaseous products was calculated to be approximately 30 days in this system. It can be mentioned that the decrease in nitrate reduction capacity can also be followed by the slow drop in the pH of the effluent from pH 8.1 at the start to pH 7.6.

Because of the decreasing nitrate reduction efficiency, we tried to activate the immobilized cells by pumping growth medium (see Materials and Methods) through the

column. This was unsuccessful because of abundant gas production within the gel which caused a considerable pressure drop in the column. Thus, the gel had to be taken out and incubated under batch conditions in growth medium at 21°C for 8 h. The gel particles swelled considerably and the increase in the reddish colour of the gel suggested that the organisms were growing within the gel matrix. After this treatment, the efficiency of the column was restored to about 97% of the initial value. The experiment was discontinued after 28 days and after this length of time the mechanical strength of the gel was still very good.

Conciusion

For the microbial denitrification of waste water somewhat similar systems based on immobilized microorganisms have been employed. In one case, a tapered fluidized cell reactor with adsorbed microorganisms was used (Scott and Hancher 1976) to remove nitrate from waste water. In another paper, nitrate- and nitrite reduction of simulated waste-water with whole cells entrapped in liquid-surfactant membranes was described (Mohan and Li 1975). Furthermore, suspended growth reactors, attached growth using anaerobic filters as well as rotating discs have also been used in microbial denitrification. Several reviews on this subject have been published (Adams 1973; Christensen and Harremoes 1977; Focht and Chang 1975).

Our studies show that pure cultures of microorganisms entrapped in alginate gel can be utilized for efficient and continuous denitrification of water. We feel that the technique described here can be used advantageously for the purification of high nitrate drinking water for the following reasons: the biocompatibility of alginate, high retention of enzymic activity after immobilization, the possibility of activation, protection of the cells in the entrapped state from external factors such as degrading enzymes or physical forces and hindered leakage of cells (in contrast to adsorbed microorganisms). Finally, the operational stability of the system has been shown to be reasonable. However, until the system can be used in practice increased mechanical stability should be achieved. To this end hardening of the beads has been attempted in preliminary tests using glutaraldehyde, but the problems of simultaneous inactivation of the entrapped microbes and increasing diffusional problems remain to be solved. Likewise, the influence of Ca^{2+} -ions on the mechanical strength of the beads will be investigated as well as the effect of Ca^{2+} -ion removing components that might be present in some high nitrate waters. In addition, alternative cheaper carbon sources will be tested and simple and reliable analytical methods for the detection of nitrate and nitrite under continuous column operation will be developed.

Acknowledgements. The financial support of The Swedish Biotechnology Research Foundation is acknowledged. The authors thank Dr. Tadhg Griffin and Dr. Robert E. Carter for linguistic advice.

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Received October 24, 1979



Columnar Denitrification of Water by Immobilized *Pseudomonas denitrificans* Cells

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Summary. Whole cells of *Pseudomonas denitrificans*, immobilized in alginate gel, were used for columnar denitrification of ground water. Ethanol was selected as a suitable carbon source and the C/N-ratio necessary for satisfactory nitrate reduction was established (1.6 mg ethanol-C/mg nitrate-N). The course of the reaction and the diffusional limitations were investigated during columnar denitrification. The mechanical integrity of the gel matrix, as judged from leakage of cells was studied. The release of cells into the effluent was effectively inhibited ($< 10^2$ cells/ml) by the use of different filter devices. The operational characteristics were determined by studying a column operating for nearly four months. Theoretically, the alginate gel column should, from high nitrate drinking water (22 mg NO_3^- -N/l), produce 3 l of denitrified water/kg gel/h (wet wt.) during a period of two months. The regeneration of nitrate reduction activity by means of activation in nutrient media proved a useful tool for restoring initial activity, the gel column having shown no loss in activity at the end of the operation period.

Introduction

In recent years, an accelerating contamination of ground water supplies with nitrate has initiated the search for effective reduction procedures (Gauntlett and Craft 1979; Adam 1980; Richard et al. 1980). In the case of waste water treatment, microbial denitrification (reduction of nitrate, via nitrite to nitrogen gas) is a well established technique (Christensen and Harremoes 1977; Hollo et al. 1980; Prakasam and Krup 1980). This method can also

be applied to the treatment of high nitrate ground waters. In a previous investigation (Nilsson et al. 1980) we used immobilized cells in microbial denitrification. In the continuation of this work, as presented in this paper, efforts were made to optimize the conditions for columnar denitrification with alginate-immobilized *Pseudomonas denitrificans* cells with the use of ethanol as the carbon source. Factors, such as selection and consumption of carbon, maintenance of gel integrity, decay and regeneration of nitrate reduction activity, are discussed.

Materials and Methods

Materials. Sodium alginate (type IV, practical grade) was obtained from Sigma Chemical Co, USA. Other reagents were of analytical grade and were purchased from commercial sources. A prefilter (pore size 10 μm) and a membrane filter (pore size 0.8 μm) from Sartorius, FRG, were used.

Cultivation of Microorganisms. Cultures of *Pseudomonas denitrificans* (ATCC 13867) were used in this study. The microorganisms were maintained and grown as previously described (Nilsson et al. 1980).

Immobilization of Cells. Centrifuged and washed (3×10 min, 12,000 $\times$ g) *P. denitrificans* cells (7 g wet wt., approximately 4×10^{11} living cells/g) were suspended in 93 g of 2% (w/v) sodium alginate. The cells were immobilized according to Nilsson et al. (1980). The cell-alginate particles were stored at +4 °C until used.

Determination of Nitrate and Nitrite. Nitrate concentrations were assayed with an ion selective electrode (Orion Research, USA, model 93–07). Nitrite concentrations were determined spectrophotometrically according to Standard Methods for the Examination of Water and Waste Water (1975).

Determination of Ethanol and Gases. Ethanol concentrations were measured either with a gas chromatograph or enzymatically. The gas chromatograph used, was a Varian 940 equipped with a teflon column (Chromosorb 101, 240 $\times$ 0.16 cm). Nitrogen was used as

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Table 1. The effect of different carbon sources on columnar denitrification with *P. denitrificans* gel^a

Carbon source	Denitrification rate (%) ^b
Potassium aspartate	100
Ethanol	58
Sodium acetate	34
Methanol	0

^a Continuous denitrification with a *P. denitrificans* gel column (15 g wet wt.), cell concentration 7% (w/v), average bead size 2.5 mm. The substrate solution (22 mg NO₃⁻-N/l, 0.37 g CaCl₂/l, C/N = 3.5) was passed upwards at 22 °C through the column with a flow rate of 2.5 ml/min

^b A denitrification rate of 100% corresponds to the consumption of 3.2 µg NO₃⁻-N/min/g gel (wet wt.) and was measured during a period of 48 h

carrier gas at 160 °C and the flow rate was 30 ml/min. The enzymatic method was based on the use of alcohol dehydrogenase (Kaplan and Ciotti 1957). Gases (N₂, CO₂ and N₂O) were measured with gas chromatography according to van Huyssten (1967).

Determination of Cell Leakage from Column. The number of living cells in the effluent from the column was estimated by viable count on nutrient agar. The colonies were counted after incubation for 22 h at 28 °C.

Results and Discussion

Selection of Carbon Source

A carbon source suitable for denitrification of ground water with immobilized cells must be cheap, non-toxic and give a high reduction rate. Furthermore, it should not interfere with the integrity of the support. The most widely used carbon source in waste water denitrification is methanol (Christensen and Harremoes 1977). Owing to the potential health hazards associated with methanol together with its apparent inability to serve as a carbon source for *Pseudomonas denitrificans* in denitrification, it was not considered further (Table 1). According to Nurse (1980), the only type of bacteria capable of utilizing methanol in pure culture denitrification is *Hyphomicrobium* spp. In our previous investigation (Nilsson et al. 1980) potassium aspartate was selected as carbon source mainly because of its high nitrate reducing power, but its use would be too costly for denitrification of water supplies on a large scale. As shown in Table 1, ethanol can be used with advantage in columnar denitrification, besides which it is cheap compared to other suitable carbon sources, such as amino acids. It should be emphasized that ethanol is used in such a low concentration (< 0.05% (w/v)) that it does not infringe upon the regulations assigned by the authorities.

Table 2. The effect of different C/N-ratios for ethanol in columnar denitrification with *P. denitrificans* gel^a

C/N-ratio (input)	Nitrate consumption (mg NO ₃ ⁻ -N/l)	Ethanol consumption (mg EtOH-C/l)	C/N-ratio (required)	C _R ^b
1.0	8.2	13.1	1.60	1.7
4.0	14.7	23.5	1.60	1.9
7.9	13.8	22.8	1.65	2.0

^a Continuous denitrification with a *P. denitrificans* gel column (15 g wet wt.), cell concentration 7% (w/v), average bead size 2.5 mm. The substrate solution (20.3 mg NO₃⁻-N/l, 0.37 g CaCl₂, ethanol (different C/N-ratios)) was passed upwards at 22 °C with a flow rate of 2.5 ml/min during a period of 24 h

^b Consumptive ratio

A certain amount of organic carbon must be present in the water to allow effective denitrification. This amount is described in terms of minimum organic carbon/nitrate nitrogen (C/N) ratio. Theoretically, when considering merely denitrification with ethanol as the carbon source the calculated C/N ratio necessary for complete reduction to N₂ is 0.71. However, in practice the carbon requirement is greater owing to deoxygenation of the water and microbial growth. To illustrate this, a consumptive ratio (C_R) was defined (McCarty et al. 1969) as the ratio of the total quantity of organic carbon source consumed to the stoichiometric requirement for denitrification and deoxygenation alone. A C_R-value higher than 1.0 indicates bacterial synthesis. We examined the effect of variation of the C/N-ratio in denitrification with immobilized cells (Table 2). We found that the required C/N-ratio for maximum reduction was 1.6–1.7 which is well above that necessary for denitrification alone (C/N = 0.71). The C_R-values obtained clearly suggest the significance of *de novo* biosynthesis in the system. In the subsequent procedures for columnar denitrification with immobilized cells, the C/N-ratio used ranged from 2.0 to 6.5, in order to secure a satisfactory supply of organic carbon.

Course of Reaction

The reaction profile of columnar nitrate- and nitrite reduction with immobilized cells was evaluated with a four column system operating in series (Fig. 1). The steady decline in nitrate concentration was accompanied by the appearance of intermediate nitrite. The course of continuous denitrification resembled that found for batch incubation with free or alginate-immobilized *Pseudomonas denitrificans* cells (Nilsson et al. 1980). These reduction patterns are also in accord with earlier findings in the field of waste water denitrification (Requa and Schroeder 1973). It is essential to

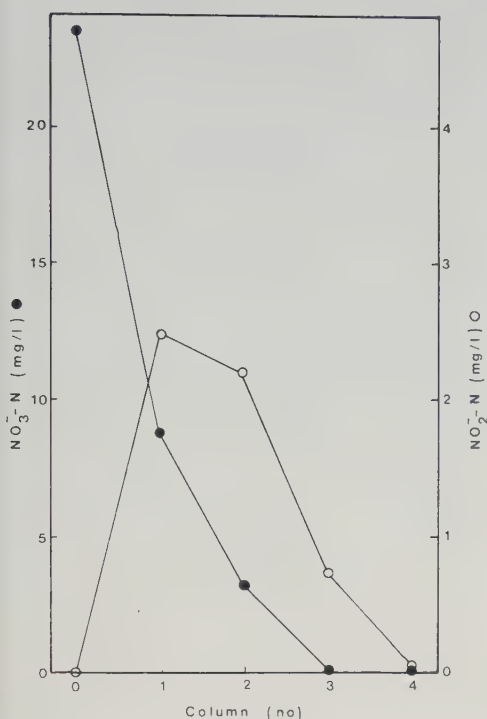


Fig. 1. The reduction profile of columnar denitrification with alginate immobilized *P. denitrificans* cells. Four identical columns, each containing 20 g of gel (wet wt, average bead size 3 mm), were connected to each other in series. The progress of denitrification was followed by collecting samples of the effluent of each column outlet. The substrate solution (23.5 mg NO₃⁻-N/l, 150 mg EtOH/l and 0.55 g CaCl₂/l) was passed continuously upwards through the columns at a flow rate of 3.2 ml/min (22 °C)

Table 3. The effect of particle size on columnar denitrification with *P. denitrificans* gel^a

Flow rate (ml/min)	Particle size (diam. in mm)		
	0.5	3.0	4.0
0.5	3.8 ^b	3.8	3.4
1.0	6.8	6.7	5.4
2.0	13.1	9.8	5.2

^a Continuous denitrification with *P. denitrificans* gel columns (1.6 g cells (wet wt.)/column, approx. 15 g gel (wet wt.)). 0.5 mm particles in form of fibers, others in form of beads. The substrate solution (19 mg NO₃⁻-N/l, 0.37 g CaCl₂/l, 150 mg EtOH/l) was passed upwards at 22 °C through the columns

^b Denitrification rate given as mg NO₃⁻-N reduced · 10²/g gel/h

emphasize that the immobilized cell column should have an overcapacity in terms of nitrate reduction in order effectively to remove the intermediate toxic nitrite formed during the latter part of the process.

Effect of Diffusional Limitations

It is well established that diffusion restricts the transport of substrates and products in alginate gels (Klein and Wagner 1979). We investigated these diffusion effects under conditions of columnar denitrification in an experiment with different-sized bacterial gels containing *P. denitrificans* cells. The results given in Table 3 on the influence of the flow rate on reduction capacity for different-sized particles, illustrate the role played by diffusion in the reduction process. No significant increase in pressure drop was observed. The above results suggest that as long as the fall in pressure is tolerable, diminution of the alginate support will increase the efficiency of columnar denitrification several times.

Mechanical Integrity of Alginate Beads

Before immobilized cells can be used on a large scale for purifying potable water, it must be shown that the purified water fills the requirements prescribed by the public health authorities. The major problem in denitrification with alginate immobilized cells is the undesired leakage of cells from the gel matrix. During continuous operation of the process the concentration of the cells in the effluent water was estimated to be about 10⁵–10⁶ cells/ml.

However, multivalent ions such as calcium, stabilizes the gel structure and thereby reduces the loss of cells from the gel surface. A significant decrease (within two orders of magnitude) was observed in the presence of calcium (0.37 g CaCl₂ · 2H₂O/l, equal to 14 °dH), a concentration not impairing the drinking quality of the water. On the other hand, chemical stabilization by treatment of alginate gel beads with cross-linking agents, such as glutaraldehyde, hexamethylenediamine and polyethyleneimine, resulted in a high loss of activity (50–100%). This loss was probably caused by the toxic effect of the organic agents on the living cells.

A more direct way to minimize cell leakage into the water would be to integrate the cell system into an isolated operating unit with an ordinary filter device. Our preliminary findings suggest that by connecting the *P. denitrificans* cell columns in series with a filter unit consisting of a prefilter (pore size: 10 µm) and a membrane filter (pore size: 0.8 µm), the leakage of cells during columnar denitrification can be reduced to a level below that required by Swedish standards for bacterial contamination of potable water (total count <10² cells/ml). The useful life of our

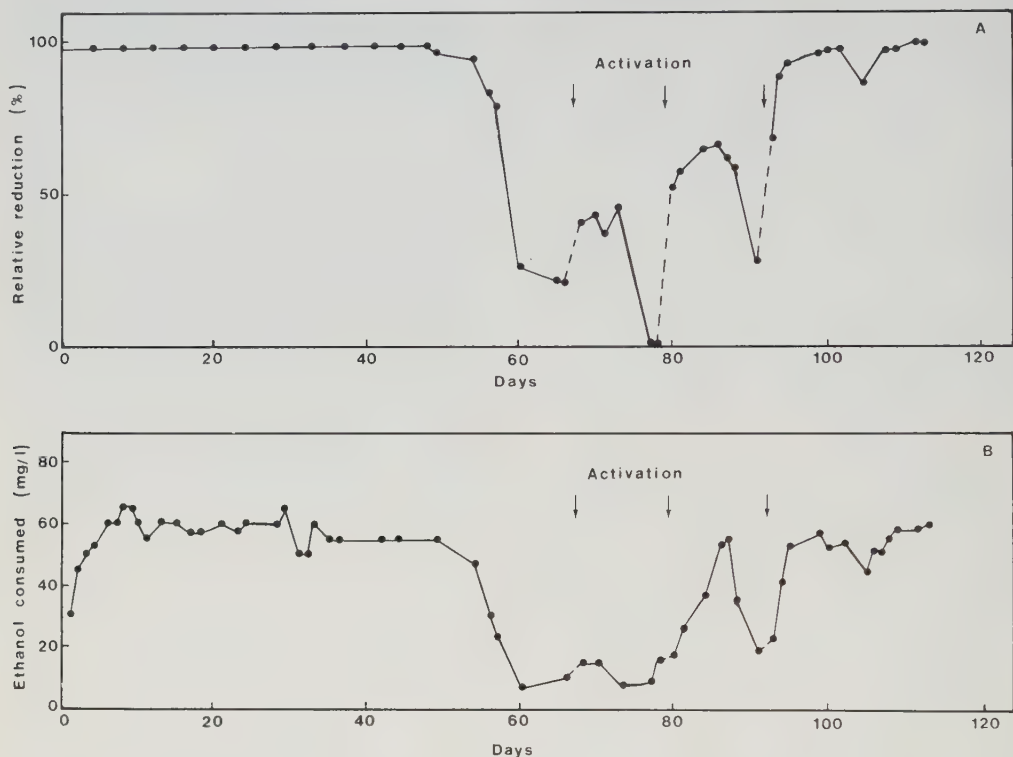


Fig. 2a and b. Columnar denitrification with alginate immobilized *P. denitrificans* cells. The substrate solution (22 mg NO_3^- -N/l, 80 mg EtOH/l and 0.37 g CaCl_2 /l) was passed upwards through the column (30 g gel (wet wt.); average bead size 2.5 mm) at 22 °C with a flow rate of 1.5 ml/min. At the times indicated by the arrows the gel was incubated at 30 °C for 20 h in growth medium containing in g/l: K-aspartate 15; yeast extract 7; peptone 7; KNO_3 8; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 0.0025; MnCl_2 0.0025; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.006; pH 6.75. The figures given in Fig. 2a denote the reduction of nitrate to gaseous products (% of inlet concentration of nitrate)

filters was rather limited (1–2 days), but by adapting the size of the filtering unit to a greater demand, an operational lifetime in the order of 1–2 months should be possible.

Operational Characteristics in Columnar Denitrification

Owing to its anaerobic character microbial denitrification is well suited for continuous operation with immobilized cells in a plug flow reactor. Some promising results of nitrate reduction with alginate immobilized *P. denitrificans* cells have already been obtained (Nilsson et al. 1980). To evaluate the prospects for immobilized cells in long-term denitrification we continued our work on these immobilized cells. Figure 2 shows an illustrative example of the performance of continuous denitrification for a period of nearly four months.

During the first 48 days of the test the operational stability was excellent with no significant loss in reduction of nitrate. It is not meaningful here to discuss the stability in terms of half-life figures, since in this case it is only apparent. Furthermore it is not possible to determine the exact loss in activity. All we can say is that the system worked satisfactorily during this initial period. After 53 days the activity fell rapidly. The reason for this loss in activity is not known in detail, but it can be speculated that during the first 53 days the rate of denitrification is under diffusion control. Furthermore, the rapid loss in activity after day 53 suggests that due to the decay of cells, the overall reaction rate is limited by the catalytic activity of the cells, i.e. the system is under reaction rate control. Several factors, such as nutrient limitations and toxic metabolites might be responsible for the decrease in the number of actively denitrifying cells. To remedy this loss we tried to activate the gel with

growth medium. Activation is an established procedure to increase the activity of immobilized cells in situ (Ohlson 1980). As shown in Fig. 2a after three successive activation periods the capacity was fully restored to its original value and maintained it at that level for the rest of the experiment.

Another parameter influencing the capacity is the C/N ratio. Ethanol, used as the carbon source, was added in an input C/N ratio of 1.9 to the high nitrate water. When the system is working at maximum rate, i.e. 98% nitrate reduction to gaseous products, we found that the required C/N ratio was 1.4 ($C_R = 1.8$) which is well in agreement with the above discussion on C/N and C_R ratios for ethanol. Figure 2b shows that the ethanol consumption was closely correlated to the reduction activity of the column. The gaseous products, containing about 95% N_2 and 5% CO_2 were collected by means of a gas trap at the top of the column. No N_2O gas was detected during the experiment. Theoretically, this unit of alginate-immobilized *P. denitrificans* cells with ethanol as the carbon source should produce, from a high nitrate drinking water (22 mg $NO_3^- - N/l$), 3 l of denitrified water/h/kg gel (wet wt.) during a period of two months. The undesired leakage of cells could be prevented by external filter devices. Complete regeneration of the cell preparation after long periods (months) of operation is possible by means of the in situ activation procedure for immobilized cells.

We feel that a treatment process based on immobilized cells, with ethanol as the carbon source, could be an effective and economical way to produce potable water from high nitrate waters.

Acknowledgements. Financial support from the Swedish Biotechnology Foundation is acknowledged. The authors are indebted to Miss Kristina Dunér, Miss Barbara Goldschmidt and Mr. Olle Holst for skilful technical assistance and valuable discussions. We also thank Mr. James Brown for linguistic advice.

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Received September 18, 1981

Biofilm Build-Up of *Pseudomonas putida* in a Chemostat at Different Dilution Rates

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Summary. A test system was set up where the build-up of a biofilm on a defined surface could be studied in a carbon source limited chemostat.

The attachment of *P. putida* ATCC 11172 to glass when growing on L-asparagine was studied at different dilution rates (specific growth rates) from 0.1 to 1.5 h⁻¹. The number of attached colony forming units (cfu) increased with dilution rate from 1 × 10⁶ cfu/cm² at 0.1 h⁻¹ to 4 × 10⁷ cfu/cm² at 1.0 h⁻¹ and then the attachment decreased to about 6 × 10⁶ cfu/cm² at higher dilution rates (1.1–1.5 h⁻¹). The number of attached cfu was measured after 24 h exposure. The value of the maximum specific growth rate in batch culture was 0.6 h⁻¹.

The total amount of attached cell-mass followed roughly the same pattern as the viable count.

The viable count of the cells suspended in the growth medium showed its lowest value at the same dilution rate as resulted in maximum adhesion.

It was shown that the effect of growth rate on the biofilm build-up of *P. putida* is significant, and ought to be borne in mind when continuous culture systems are set up and results evaluated.

the physiological status of the cell which, in turn, depends on environmental parameters, e.g. nutritional conditions, pH and temperature (Fletcher 1977; Wood 1980).

These effects ought to be taken into account when industrial fermentation processes based on biofilms are to be designed and when the conventional continuous culture is used as a research tool in the laboratory. In the former case, the attached growth is encouraged while it is discouraged in the latter one as it could give rise to considerable deviations from the theoretical growth models.

Pseudomonas putida is an organism of considerable biotechnical interest. Thus, it has a potential for fast growth on nutritionally poor substrates and has a remarkably high metabolic versatility. The latter is reflected in its ability to utilize a wide spectrum of carbon sources for growth (Molin and Ternström 1982). Furthermore, *P. putida* may be used in detoxification processes. For example, some strains are able to grow on toluene and to oxidize 3-methylcatechol and chlorobenzene (Klecka and Gibson 1981).

P. putida ATCC 11172, chosen as test organism in the present study, grows fast on simple media and efficiently forms biofilms (Molin 1981). This organism is also able to grow on p-cresol as sole carbon source (Hatt et al. 1976).

The intention of the present paper was (1) to develop a test system where the influence of different growth conditions on the build-up of a biofilm could be studied in a chemostat, and (2) to use this system, primarily, to evaluate the influence of growth rate (dilution rate) on the attachment of *Pseudomonas putida* ATCC 11172.

Materials and Methods

Microorganism. Cultures of *Pseudomonas putida* ATCC 11172 were maintained freeze-dried in skim-milk at +3 °C. Strain ATCC

Introduction

The importance of attached growth of microorganisms in natural environments as well as in continuous fermentation processes has received much attention in recent years (Atkinson and Fowler 1974; Ash 1979; Fletcher 1980; Andrews and Chi Tien 1981; Messing 1981). The attachment process and the development of a biofilm depends to a great extent on genetical inherent features of the bacterial strain. However it is also highly dependent on

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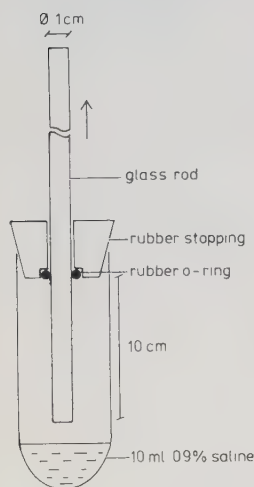


Fig. 1. The sampling device for desorption of an attached cell film from a defined surface

11172 is in the American Type Culture Collection (Hatt et al. 1976) labelled as *P. fluorescens* but the organism was reclassified as a typical *P. putida* biotype I by Molin and Ternström (1982).

Media and Inoculation Procedures. The freeze-dried culture was recovered in two steps on a rotary shaker at 25 °C; (I) the culture was transferred into 200 ml of nutrient broth (Difco, USA) and incubated for 8 h; (II) 5 ml of the broth was transferred into 100 ml of medium of the following composition (g/l): L-asparagine (monohydrate; 1566, Merck), 5.0; MgSO_4 , 0.50; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.40; KH_2PO_4 , 0.37; $(\text{NH}_4)_2\text{SO}_4$, 0.25; CaCl_2 , 0.1; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.05; citric acid, 0.02; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.005; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005; pH = 6.7. This culture was incubated for 17 h and was then used as inoculum in the fermentor.

The fermentor medium was equal to that mentioned above except that it contained 3.0 g/l asparagine and 0.10 ml/l Silicone antifoaming agent (BDH Chemicals Ltd, England). The continuous flow was started at an optical density of about 0.6. The asparagine content in the medium for continuous culture was lowered to 1.0 g/l. The pH of this medium was before sterilization adjusted to 4.0 by addition of orthophosphoric acid.

Equipment. The experiments were accomplished in a 4 l fermentor (Chemoferm AB, Sweden) with a working volume of 2.5 l. The medium was fed by a membrane pump (Precision dosage pump Fe 211, Braun Melsungen, FRG) from a 125 l steam sterilizable medium tank (Chemoferm AB, Sweden) and the outflow was maintained via an overflow-outlet and a peristaltic pump. The culture was sparged with 2.5 l air per min and the stirrer speed was 750 rpm. The dissolved oxygen tension was measured using a galvanic type of oxygen electrode (Johnson et al. 1964). The pH was maintained by titration with 0.6 M HCl (pHM 62 + TTT 60, Radiometer, Denmark). The carbon dioxide in the outlet gas was registered using a Binos 1 IR Gas Analyzer (Leybold Heraeus, FRG).

To determine the biofilm development glass rods (diameter 10 mm) were immersed into the growing culture. The rods were

pretreated with hydrofluoric acid (7.5 M, 45 min) and were marked at a height of 10 cm to define the area to be examined.

Biofilm Experiments. *P. putida* was grown in a continuous culture under carbon source limitation at pH 6.7 and 25 °C. The culture was run at different dilution rates ($D = 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3, 1.5$ and 0.5 h^{-1} , in sequence) for a total period of 21 days. When the culture was in steady-state ($3 \times$ residence time, 3–30 h at different dilution rates) measurements were made of optical density (OD_{620}), viable count (Tryptone glucose extract agar, Oxoid, 3 d, 25 °C), L-asparagine (enzymatically UV-test, Lebensmittelanalytik, Boehringer, Mannheim, FRG), dry weight, carbon dioxide concentration in the outlet gas and dissolved oxygen tension of the medium.

To evaluate the development of a microbial film at different dilution rates, pretreated and sterilized glass rods were at steady-state immersed into the fermentor and exposed to the growing culture for different periods of time (0.5 min – 72 h). After exposure, the rods were removed from the fermentor and inserted into a scraping device (Fig. 1) where the biofilm was transferred to a rubber O-ring. To be easily applied, the O-ring and the rubber stopper were cut open. After detachment, the O-ring holding the biomass, was dropped into 10 ml of 0.9% saline. The tube was vigorously shaken using a vortex mixer, after which a conventional viable count was performed (TGE-agar, 3 d, 25 °C). The total attached cell mass measured as the optical density (OD_{620}) of the solution, was also determined.

At the start of all measurements one glass rod was immersed for 0.5 min thereby giving the initial number of bacteria associated with the surface or surface liquid.

Results

The adhesion of *P. putida*, to a defined glass surface, was studied in a continuous culture with L-asparagine as the only limiting carbon and energy source.

The method developed to estimate the amount of attached growth was tested for reproducibility by simultaneously immersing three glass rods into the fermentor for 33 h at a dilution rate of 0.7 h^{-1} . The resulting viable counts were 1.0×10^8 , 0.96×10^8 and 0.99×10^8 colony forming units (cfu)/ cm^2 . The corresponding values of the optical density of the 10 ml saline were 0.375, 0.355 and 0.360, respectively.

Figure 2a shows the attachment to the glass rods after 24 h exposure at different dilution rates. There was a maximal attachment around the dilution rate $D = 0.9 \text{ h}^{-1}$ where the viable count reached 4×10^7 cfu/ cm^2 . At about the same dilution rate there was a marked minimum in the concentration of suspended viable cells (Fig. 2b). The viable count of the suspended cells decreased from about 3.5×10^8 ($D = 0.7 \text{ h}^{-1}$) to 1×10^7 cfu/ cm^2 ($D = 0.9 \text{ h}^{-1}$).

To control the measurements in Fig. 2a a glass rod was immersed into the culture for 30 s at each dilution rate and then measured for viable count. These initial viable count values were about the same (5×10^5 to 1×10^6 cfu/ cm^2) for all the tested dilution rates. The control values were apparently not dependent on the suspended cell concentration.

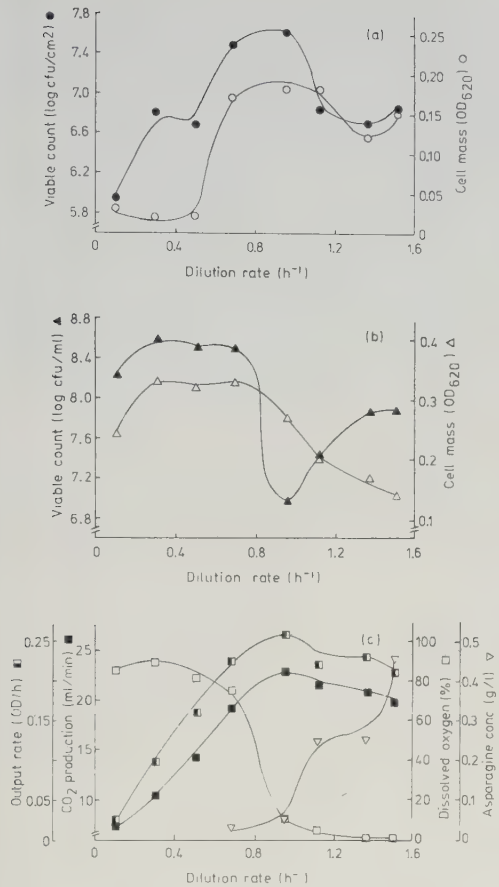


Fig. 2a–c. Continuous growth of *P. putida* with L-asparagine as the only carbon source. **a** attached growth after 24 h exposure: (●) colony forming units per cm^2 ; (○) cell mass as OD_{620} -values. **b** suspended growth: (▲) colony forming units per ml; (△) cell mass as OD_{620} -values. **c** overall activity: (■) produced CO_2 ; (●) output rate; (▽) asparagine concentration; (□) dissolved oxygen

Table 1. Influence of exposure time on the biofilm build-up at different dilution rates. The biofilm is measured as colony forming units (cfu) per cm^2 of glass surface

Exposure time (h)	Dilution rate (h^{-1})		
	0.30	0.50	0.70
0.01	5.9 ^a	5.9	5.6
7.0	6.1	6.1	6.8
24.0	6.8	6.7	7.5

^a Numbers of attached cells are given as $\log \text{cfu per cm}^2$

Figure 2b shows the steady-state characteristics of the culture. It can be seen that the suspended cell-mass is maintained at a rather high value above the critical dilution rate as indicated by the maximum specific growth rate of a batch culture (0.59 h^{-1}). The productivity, given as output rate, is also maintained at a high level at high dilution rates (Fig. 2c).

It should be pointed out that at the high dilution rates (above 1.2 h^{-1}), where the suspended cell-mass is decreasing, the attached cell film and the concentration of suspended colony forming units remain stable (Figs. 2a and 2b).

The size of the single suspended cell of the culture was successively increasing with increasing dilution rate. The cells were more than three times larger at the highest dilution rate than they were at the lowest.

The build-up rate of the attached cell film at three different dilution rates is shown in Table 1. It can be seen that the build up rate during the first 7 h was considerably higher at $D = 0.7 \text{ h}^{-1}$ than at the lower dilution rates. Between 7 and 24 h the increase in cell film proceeded at a similar rate for all the tested dilution rates.

Discussion

The adhesion of microorganisms to defined surfaces has mostly been studied either in batch cultures or in different kinds of uncontrolled continuous cultures of mixed populations (Atkinson and Fowler 1974; Ellwood et al. 1979). The environmental conditions of both types of systems are poorly defined. The experimental set-up of the present study provides the means to follow the adhesion of cells in a chemostat under pure culture conditions.

The curve representing the suspended cell-mass (optical density) versus dilution rate has a somewhat unconventional appearance, i.e. the cell-mass concentration remains high far above the theoretical critical dilution rate of the culture and is then only declining slowly at higher dilution rates. This is due to attached growth on the fermentor walls and equipment and has been demonstrated earlier for the same organism growing on the same medium in a chemostat (Molin 1981). There were some differences in curve shape between this earlier study and the present one, which was probably due to differences in fermentor volume (1.0 l and 2.5 l) and, hence, the difference in volume/surface ratio (about 1 ml/cm^2 and 2 ml/cm^2 , respectively).

A pronounced characteristic of the culture was the minimum of suspended viable cells at $D = 0.95 \text{ h}^{-1}$ (Fig. 2b). A similar minimum at the same dilution rate was found by Molin (1981). Thus, this somewhat surprising characteristic is obviously reproducible and is probably reflecting some sort of physiological event in the culture. It is striking that at the same dilution rate the attachment capacity of the culture is at its highest level (Fig. 2a).

The maximal attached cell density obtained on glass after 24 h exposure in the present study was about 4×10^7 cfu/cm². This value can be compared with some literature values for *Pseudomonas* (cfu/cm²), e.g. 4×10^7 (polystyrene; Fletcher 1977), 10^3 – 10^5 (glass; Wardell et al. 1980), 6×10^7 (spoiled meat; Blickstad et al. 1981) and 6×10^8 (spoiled meat; Erichsen and Molin 1981). The densities obtained by, for example, Enterobacteriaceae seem to be of a similar magnitude (Munson and Bridges 1964; Wardell et al. 1980). Thus, the capability of *P. putida* in the present study to form a dense viable cell film is comparably good and the density values obtained could be compared with the theoretical maximal concentration of a monolayer of *Pseudomonas* cells. By definition a *Pseudomonas* cell should be 0.5×1 to $2 \times 4 \mu\text{m}$ in size, i.e. the maximal density of a monolayer must be somewhere in the range 1×10^7 to 2×10^8 cells/cm².

On the other hand, the capacity of an organism to adhere to surfaces is not only defined by the maximal obtained cell density but also by the rate of film development. In the present study the rate of build-up was dependent on the dilution rate. At a dilution rate of 0.5 h^{-1} the cell film increased during the first 7 h of development by about $0.02 \log \text{ cfu/cm}^2$ and h. The corresponding value at 0.7 h^{-1} was $0.2 \log \text{ cfu/cm}^2$ and h (Table 1). Fletcher (1977) reported that pseudomonad cells, harvested in the exponential phase of a batch culture, adhered to polystyrene at a rate of about $0.05 \log \text{ cfu/cm}^2$ and h. Thus, the latter value fits in between those of the present study. However, polystyrene is known as a more favourable adhesion material than glass (Fletcher and Loeb 1976).

The attachment capacity of the culture at different dilution rates was indicated in Fig. 2a, i.e. these values reflected the overall attachment after a 24 h period. The attachment successively increased with dilution rate up to about 1.0 h^{-1} and up to this value the attachment was increasing with increased growth rate as the growth rate is equal to dilution rate in a chemostat. Thus, the faster the growth rate the better the attachment. This is in agreement with data reported by Fletcher (1977) who showed that the cells in a batch culture had higher attachment capacity in the exponential growth phase than in the lag or declining phase.

It is a general belief that bacterial activity in low nutrient environments is enhanced at surfaces, i.e. an environment where the carbon source is limited would stimulate adherence (Atkinson and Fowler 1974; Fletcher 1979). This hypothesis is, for example, supported by the results of Marshall et al. (1971) and Brown et al. (1977) who showed that attachment could be inhibited by an excess of carbon source (glucose). The adhesion of *P. putida* in the present study was good throughout the experimental series which could be expected on the basis of the above mentioned hypothesis and the fact that the chemostat was carbon source limited. However, the general input of

carbon source into the system increased as the dilution rate increased and simultaneously the attachment capacity of the culture also increased (up to about 1.0 h^{-1}). Furthermore, in spite of the large excess of carbon source at higher dilution rates, the attachment capacity of the culture was maintained on a fairly high level (Fig. 2a). In consequence, the amount of carbon source per se did not seem to have a critical influence on the attachment. The growth rate seems to be a more important factor in the present study.

In conclusion, the effect of specific growth rate (dilution rate) on the adherence of *P. putida* is a phenomenon that ought to be taken into account not only in laboratory fermentor studies where attached growth is mostly a disadvantage, but also in the designing of industrial biotechnical processes where attached growth can be utilized advantageously.

Acknowledgements. The authors would like to express their gratitude to Dr. Milan Dostálek and Professor Nils Molin for valuable discussions.

This study was supported by The Swedish National Board for Technical Development.

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Received June 4, 1982

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Effect of Different Environmental Parameters on the Biofilm Build-Up of *Pseudomonas putida* ATCC 11172 in Chemostat

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Summary. The influence of carbon source, cell concentration, oxygen tension, pH and temperature on the biofilm build-up of *Pseudomonas putida* ATCC 11172 was studied. The experiments were performed in a carbon and energy limited chemostat (asparagine). When the asparagine was replaced by glucose the biofilm build-up was decreasing.

Cell concentration, oxygen tension or temperature did not to any significant degree affect the biofilm build-up. Temperature, however influenced the size of the suspended cells. The cell size successively increased with decreasing temperature. A similar change in cell size could be accomplished by increasing the dilution rate from 0.2 h^{-1} (small cells) to 0.7 h^{-1} (large cells).

The biofilm build-up increased with increasing pH in the interval of 5.5–6.7. The viable count of the biofilm after 24 h exposure of test surface increased from $8 \times 10^6 \text{ cfu/cm}^2$ to $1.6 \times 10^8 \text{ cfu/cm}^2$ while the biomass increased by a factor of about 70.

collect data concerning which and to what extent different environmental parameters affect the biofilm build-up and how they function. This may be of special interest with regard to *Pseudomonas*. However, there is a sparsity of data in the literature and moreover, much of that data comes from studies where the growth rate has been uncontrolled, i.e., the effect of a certain parameter has been studied without concern for the fact that changes in the parameter probably influence the growth rate which, in turn, may influence the biofilm build-up. It has been shown, by Molin (1981) and Molin et al. (1982), in a chemostat that the biofilm build-up is influenced by the dilution rate (growth rate).

The present study deals with the influence of carbon source, cell concentration, oxygen tension, pH and temperature on the biofilm build-up of *Pseudomonas putida* ATCC 11172. This study was performed in a carbon and energy limited chemostat (asparagine) and, thus, under controlled growth rate.

Introduction

Biofilm formation and the adhesion of microorganisms to surfaces are phenomena that have merited much interest in recent years (Messing 1981; Ellwood et al. 1982). One reason for this is the growing awareness that biofilms can advantageously be used in biotechnological processes (Atkinson and Fowler 1974). Another is its importance for the microbiological ecology in the natural environment (Fletcher 1979).

One biotechnical application for biofilm based processes is waste water treatment, which, in aerobic applications is a process where the genus *Pseudomonas* is deeply involved. Thus, it is of interest to

Materials and Methods

Micro-organism

Cultures of *Pseudomonas putida* ATCC 11172 were stored freeze-dried in skim-milk at $+4^\circ \text{C}$. This organism is in the American Type Culture Collection (Catalogue of strains I, 1982) labelled as *P. fluorescens* but was reclassified as a *P. putida* biotype I by Molin and Ternström (1982).

Media, Inoculation Procedures and Equipment

The freeze-dried cultures were recovered and grown in a carbon source limited chemostat using inoculation procedures and media as previously described (Molin et al. 1982). The equipment was also identical with the earlier set up. The only exception was that the dissolved oxygen tension of the medium in the present study was measured using a sterilizable polarographic oxygen electrode (Radiometer, Denmark).

Continuous Culture

P. putida was cultured in a carbon source limited chemostat. When steady-state was obtained (more than 3 times the residence time), measurements were made of the viable count (TGE-agar, Oxoid, 3 days, 25° C), absorbance (A_{620}), L-asparagine and L-asparaginic acid or D-glucose (enzymatically UV-test, Lebensmittelanalytik, Boehringer, Mannheim, FRG), dry weight, dissolved oxygen tension of the medium and carbon dioxide concentration in the outlet gas.

Biofilm Experiments

The development of the microbial film was evaluated using glass rods immersed into the fermentation broth. The sampling device and procedures were previously described (Molin et al. 1982). This procedure gave an estimation of the biofilm formed in terms of colony forming units (cfu)/cm² of glass surface and total amount of attached biomass, measured as A_{620} , of a suspension of the detached biofilm.

The effect of different environmental parameters on the biofilm build-up was unless otherwise is stated, studied at the dilution rate (D) of 0.7 h⁻¹; 25° C; pH of 6.7; gas flow of 1.0 VVM; 1.0 g L-asparagine per liter medium. The different parameters studied were (I) the dissolved oxygen tension, (II) the carbon concentration and source, (III) the pH and (IV) the temperature.

(I) *Dissolved Oxygen Tension.* The *P. putida* culture was run, in sequence, at the following dissolved oxygen tensions, 50%, 10%, 0.5%, 50%, 10%, and 0.5% of saturation. In order to reach these oxygen tensions, the inlet gas flow (1.0 VVM) was composed of different mixtures of air and nitrogen.

(II) *Carbon Concentration and Source.* *P. putida* was grown as described under (I) with 2.5 l air/min, except that the L-asparagine concentration was changed at intervals from 1.0 g/l, 0.5 g/l, 2.0 g/l, to 0.5 g/l and 2.0 g/l (in sequence).

To compare the biofilm development when using different carbon sources, L-asparagine (1.0 g/l) was in one separate experiment replaced by an equivalent concentration of glucose (1:1, carbon to carbon). The loss of nitrogen by substituting asparagine was replaced by an increase of ammonium sulfate in the medium. This experiment was otherwise run under "standard conditions".

(III) *pH.* The effect of different pH-values on the biofilm development was estimated. The chemostat was run at three different pH, in sequence, 5.5, 6.0, 6.7, and 5.5.

(IV) *Temperature.* The effects of different growth temperatures on the biofilm formation were studied in sequence at 25° C, 17° C, 10° C, 25° C, 17° C, and 10° C. In order to maintain the carbon source limitation the dilution rate was kept low at 0.2 h⁻¹ throughout the experiment.

Where applicable all data given in the Tables and Figures represent the mean value of two separate experiments.

Microscopy

Viable cell suspensions withdrawn from the fermentor were directly examined in a phase-contrast microscope in order to estimate the individual cell size at different growth conditions.

Results

The effect of dissolved oxygen tension on the suspended biomass and on the biofilm build-up in a chemostat with *Pseudomonas putida* ATCC 11172 growing on asparagine as the only and limiting carbon and energy source, is shown in Table 1. The biofilm build-up on glass surfaces was constant, irrespective of the oxygen concentration, at both 6 h and 24 h of exposure to the culture. The suspended biomass was also constant when measured as absorbance (A_{620}). However, the viable count of the suspended biomass fell with the decrease in oxygen level.

When for estimating initial adhesion, the sampling surfaces for biofilm measurement were immersed for 30 s, about $4-8 \times 10^5$ cfu/cm² were attached to the surface.

Table 2 shows that there was no significant effect on the biofilm build-up of the carbon source concentration of the substrate i.e. of concentration of biomass in the fermentor. However, the biofilm build-up decreased when changing carbon source from asparagine to glucose. As a control, a sampling surface was immersed for 30 s at each test condition.

Table 1. Effects of different dissolved oxygen tensions on suspended biomass and biofilm build-up of *Pseudomonas putida* ATCC 11172 in chemostat (24 h exposure of test surface; dilution rate 0.7 h⁻¹)

Dissolved oxygen tension ^a (% of saturation)	Suspended biomass		Biofilm build-up after 24 h exposure		Asparagine in effluent (g/l)
	cfu/ml	A_{620}	cfu/cm ²	A_{620}	
50	1.2×10^8	0.28	2.0×10^8	0.24	0.02
10	9.0×10^7	0.28	2.0×10^8	0.27	0.02
0.5	1.4×10^7	0.28	1.0×10^8	0.26	0.02

^a Temperature, 25° C; pH, 6.7; carbon source, 1.0 g/l asparagine

Table 2. Effects of different substrate concentrations on suspended biomass and biofilm build-up of *Pseudomonas putida* ATCC 11172 in chemostat (24 h exposure of test surface; dilution rate 0.7 h⁻¹)

Carbon source in medium ^a (g/l)	Suspended biomass		Biofilm build-up after 24 h exposure		Carbon source in effluent (g/l)
	cfu/ml	A_{620}	cfu/cm ²	A_{620}	
Asparagine 0.5	4.3×10^7	0.11	8.2×10^7	0.50	0.01
Asparagine 1.0	1.0×10^8	0.28	3.0×10^8	0.53	0.02
Asparagine 2.0	9.4×10^7	0.51	2.8×10^8	0.45	0.02
Glucose 0.8	5.0×10^7	0.22	4.1×10^7	0.18	0.08

^a Temperature, 25° C; pH, 6.7

8×10^5 cfu/cm² were attached to the surfaces at different substrate concentrations.

Table 3 demonstrates the effect of pH on the film build-up. The higher the pH, the more dense the biofilm. The suspended biomass was not substantially influenced by the change in pH even if the content of asparagine in the fermentor increased at the lowest pH. Sample surfaces immersed in the culture for 30 s became attached by $1-5 \times 10^5$ cfu/cm².

The biofilm build-up at the different pH values for different exposure times is shown in Fig. 1a and b. It was indicated that the rate of build-up, as well as the final film density, was affected by pH. In all cases, the film density reached an equilibrium after about 24 h where no further build-up was noted. The exception was when the total biomass of the biofilm was measured at pH 6.7 (Fig. 1b). In this case, the build-up proceeded for more than 2 days.

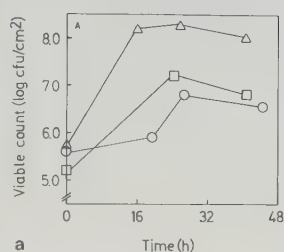
Table 4 shows the effect of temperature on the biofilm build-up at the dilution rate of 0.2 h^{-1} . There was

no difference in biofilm build-up between 25°C and 17°C and only a very slight difference in cfu/cm² between 17°C and 10°C. However, it should be noted that the viable count of the suspended cells was significantly affected, i.e., the viable count decreased at decreasing temperatures while the biomass remained constant. 2×10^6 cfu/cm² were attached to the control surfaces immersed at 17°C and 25°C. The

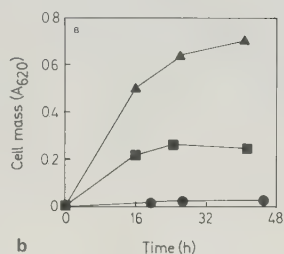
Table 3. Effects of different pH on suspended biomass and biofilm build-up of *Pseudomonas putida* ATCC 11172 in continuous culture (24 h exposure of test surface; dilution rate 0.7 h^{-1})

Suspended biomass		Biofilm build-up after 24 h exposure		Asparagine in effluent (g/l)
cfu/ml	A ₆₂₀	cfu/cm ²	A ₆₂₀	
2.0×10^7	0.16	8.0×10^6	0.05	0.70
1.0×10^7	0.26	2.5×10^7	0.27	0.03
1.1×10^7	0.24	1.6×10^8	0.63	0.02

temperature, 25°C; carbon source, 1.0 g/l asparagine



a



b

Fig. 1 a and b. Biofilm build-up of *Pseudomonas putida* ATCC 11172 growing in continuous culture at the dilution rate of 0.7 h^{-1} . a biofilm build-up measured as cfu/cm² at (○) pH = 5.5; (□) pH = 6.0; (△) pH = 6.7; b biofilm build-up measured as absorbance at 620 nm at (●) pH = 5.5; (■) pH = 6.0; (▲) pH = 6.7

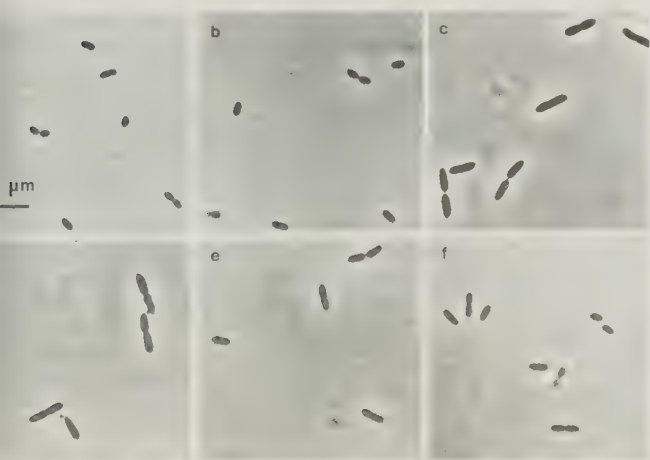


Fig. 2 a-f. *Pseudomonas putida* ATCC 11172 in carbon source limited chemostat. Bar representing 5 μm applies to the whole figure. Top: culture growing at 25°C at the dilution rates of a 0.1 h^{-1} , b 0.2 h^{-1} , c 0.7 h^{-1} . Bottom: culture growing at the dilution rate of 0.2 h^{-1} at d 10°C; e 17°C; f 25°C

Table 4. Effects of different temperatures on suspended biomass and biofilm build-up of *Pseudomonas putida* ATCC 11172 in chemostat (48 h exposure of test surface; dilution rate 0.2 h^{-1})

Temperature ^a (°C)	Suspended biomass		Biofilm build-up after 48 h exposure		Asparagine in effluent (g/l)
	cfu/ml	A ₆₂₀	cfu/cm ²	A ₆₂₀	
10	2.0×10^7	0.24	2.0×10^6	0.05	0.01
17	4.5×10^8	0.25	7.3×10^6	0.04	0.01
25	2.0×10^9	0.26	6.0×10^6	0.02	0.01

^a pH, 6.7; carbon source, 1.0 g/l asparagine

corresponding value at 10°C was $2 \times 10^5 \text{ cfu/cm}^2$.

The temperature did not only affect the viable count of the suspended biomass, it also affected the size of the cells. In Fig. 2 it is shown that the size increased at decreasing temperatures. The change in size caused by temperature could be compared to the change induced by dilution rate (growth rate). It is shown in Fig. 2 that the size increased along with an increasing dilution rate.

Variations in cell concentration, oxygen tension or pH did not seem to affect the cell size to any significant degree.

Discussion

The dilution rate of a chemostat is equivalent to the growth rate of the cells. Thus, the influence of different environmental parameters on cell physiology can be studied without interference of the growth rate, i.e., the culture can be run at a chosen, fixed, growth rate. In the chemostat of the present study the relationship between dilution rate and growth rate is also influenced by the attached growth in the fermentor, especially at higher dilution rates (Molin et al. 1982). However, the deflection of the culture from the theoretical behaviour has not to any significant degree changed the presumption that the present physiological observations have been made without interference of changes in growth rate. A dilution rate of 0.7 h^{-1} was mostly applied in the present study. This rate was chosen as it enables a fast biofilm build-up (Molin et al. 1982).

The physiological feature primarily examined in the present study was the biofilm build-up on glass during a 24 h period. This measurement is probably, but not necessarily, related to the adhesion of organisms to the clean surface. However, it is surely related to the capacity of the cells to form a biofilm

and it gives an idea of the rate of biomass build-up (Fig. 1b) as well as an indication of the final biological density of the film (cfu/cm^2 ; Fig. 1a).

A slight oxygen limitation of the culture had effect on the biofilm formation (Table 1). However, it should be pointed out that this was a weak limitation as it could not be noticed by a decrease in the total, suspended, biomass but only as a drop in the viable count. Nevertheless, the results indicate that at a starting deficiency of oxygen, the suspended cells are the primary target while the biofilm seems to keep up more efficiently with the deficient environment. Additionally, it may be stressed that due to diffusional limitations there are considerable differences in oxygen as well as substrate availability within the biofilm (Howell and Atkinson 1976).

No significant effect of substrate concentration on the biofilm build-up was observed in the present study (Table 2). However, the present study was performed in a carbon source limited chemostat and thus, irrespective of the carbon concentration of the medium the cells grew in an environment of a very low carbon source concentration. The carbon source concentration of the medium only controlled the carbon concentration in the fermentor. It is otherwise well known that an excess of carbon source prevents attachment (Marshall et al. 1971; Brown et al. 1972; La Motta 1976).

Fletcher (1977) reported that the attachment rate of a marine pseudomonad increased with increasing cell concentration, which disagrees with the present findings. However, Fletcher's culture was non-growing and the exposure period was only 2 h. Thus, what actually happened during this initial period of exposure may be insignificant for the subsequent biofilm build-up.

One important factor in the mechanism of adhesion of micro-organisms is the electrical charge of the different components in the micro-environment. If bacteria are to adhere to a solid surface they must overcome the electrostatic repulsion barrier formed by negatively charged functional groups on the cell as well as on the solid surface. The surface of electro-negatively drops with pH due to the protonation of exposed functional groups. Thus, it is not very surprising that biofilm build-up could be influenced by pH. For example, Wilkinson and Hamer (1974) have noticed that in a mixed culture dominated by a *Pseudomonas* sp there was a considerable build-up of a visible biofilm at pH above 5.8 where no such film occurred below pH 5.8. In the present study it was shown that the ability to form biofilm increased with an increasing pH and at pH 5.5 there was a drastic decrease in the total biomass of the film. However, the lack of biofilm formation at pH 5.5 may not only be due to the pH. It may have also been

caused by the fact that the culture was no longer carbon source limited (Table 3). Others have shown that an excess of carbon source prevents attachment of microorganisms to surfaces (Marshall et al. 1971, Brown et al. 1977, La Motta 1976). On the other hand, the effect of pH on the biofilm formation still holds true with respect to the stepwise decrease from pH 6.7 to pH 6.0 (Table 3).

When measuring the biofilm build-up at different exposure times we found that it reached an equilibrium after 24 h in all cases but one (Fig. 1a and b). At pH 6.7 the total biomass continued to increase after 24 h, while the viable fraction was decreasing. In this rather dense biofilm the ratio of non viable to viable cells had altered considerably. In these experiments we have not measured the actual film thickness, but it is known (Atkinson and Fowler 1974) that the active film thickness or "nutrient penetration depth" is in the ratio 50–100 μm . Films exceeding this thickness do show changes in the ratio non viable to viable cells due to substrate and/or oxygen limitation in the lower regions of the film.

No significant effects of temperature on the biofilm build-up were noticed (Table 4). In contrast, Fletcher (1977) reported that the attachment rate of a "psychrophilic" pseudomonad was lower at 3° C than at 25° C (2 h of exposure of non-growing cells).

Even if the biofilm build-up in the present study did not seem to be affected by the temperature, there was a marked physiological influence on the suspended cells. Thus, the number of viable cells per ml decreased with decreasing temperature. At the same time, the total biomass stayed constant but the size of each single cell increased as the temperature decreased (Table 4 and Fig. 2).

In the literature reports on the effect of temperature on cell size are somewhat contradictory. It has been stated that cell size increases with decreasing temperatures (Lamanna 1940) but it has also been argued that there is little or no influence (Schaechter et al. 1958, Shehata and Marr 1975) and, furthermore, that the cell size decreases with decreasing temperature (Trueba et al. 1982). In this last study, which strongly contradicts the present results, *E. coli* was tested in batch cultures in the temperature range of 22–37° C. The differences in results between this study and the present one can, of course, be due to the different test organisms used. However, it could also be due to the fact that the temperature effect on *E. coli* was tested under different growth rates. In fact, the present results show a reversed growth rate effect on the cell size of *P. putida*, i.e., the cell size increased at increasing growth rate (Fig. 2).

It should be pointed out that the size of bacteria subjected to starvation could drastically decrease (Dawson et al. 1981; Kjelleberg et al. 1982). In the

present chemostat the input of carbon and energy source decreased at decreasing dilution rate.

In conclusion, it should be noted that the only environmental parameter tested that significantly affected the biofilm formation was the pH. This factor had a specific effect on the biofilm without noticeably influencing the suspended cells. In contrast, the other environmental parameters did not affect the biofilm build-up but affected the suspended cell mass. The above could be of some importance when biotechnical processes based on biofilms are to be evaluated or designed.

Acknowledgment. The authors wish to thank Ms Lena Stenson Holst for excellent technical assistance and Dr. Milan Dostálek and Professor Nils Molin for their valuable participation in discussions. This work was financed by the Swedish National Board for Technical Development.

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Received May 13, 1983

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ESTIMATION OF THE BIOFILM FORMATION ABILITY OF PSEUDOMONAS PUTIDA
IN DISCRETE SAMPLES FROM CONTINUOUS CULTURE

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Running title: Biofilm formation ability of Pseudomonas putida

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SUMMARY

Test systems were set up in order to evaluate the ability of biomass, from continuous culture, to form biofilms. A film-forming strain of Pseudomonas putida was used as the test organism. The adsorption of resting cells onto glass surfaces was measured in specially designed chambers containing 1 ml of cell suspension. Both the quantity and the physiological activity of the adsorbed cells, in terms of optical density after detachment and pH change of a substrate exposed to the adsorbed cells, were measured. The analysis of biomass from continuous cultures of Pseudomonas putida verified the suitability of the methods. Furthermore, other properties of importance to biofilm formation, hydrophobicity and flocculation capacity of the cells, were investigated.

It was shown, for samples deriving from different dilution rates, that the cell adsorption rate increased drastically at dilution rates higher than the $\mu_{\max}$ of the culture. Simultaneously, higher values of hydrophobicity and flocculation capacity were observed. These results do shed some light on the significant biofilm build-up previously exhibited by Pseudomonas putida at higher dilution rates.

It was also shown that the age and thickness of the subsequently produced biofilm in the continuous culture influenced the specific activity. Test surfaces exposed for 44 h to the culture showed a total attached biomass five times that of a surface exposed for only 6 h. However, the specific activity of the cells in the more developed biofilm was only 20% of the activity of the 6 h biofilm.

These methods facilitate the study of parameters important to biofilm formation as well as the specific activity of the attached biomass.

INTRODUCTION

The adhesion of microbial cells and the subsequent biofilm formation on submerged surfaces has many implications, both beneficial and detrimental, in nature as well as in industry (Atkinson and Fowler 1974; Ash 1979; Fletcher 1980; Messing 1981; Atkinson 1983). Previously, the major applications using attached micro-organisms were different waste water treatment processes (trickling filters, rotating biological contactors etc.). However, the use of attached biofilms in other biotechnical processes has been intensified and many examples have been presented e.g., methane production (Fynn and Whitmore 1982), production of cell mass from alkanes (Sprenger and Rehm 1983) and ethanol production using Zymomonas mobilis (Bland et al. 1982). The development of microbial biofilms can also give rise to various disadvantages like heat transfer reduction, increases in fluid frictional resistance and material deterioration (Duddridge et al. 1982).

The mechanisms involved in the adsorption of cells onto a clean surface and the subsequent build up of a microbial film, are very complex and many factors are of importance (Bryers and Characklis 1982). The attachment is sometimes mediated by microbial surface appendages, but more commonly by extra cellular polymeric material. There is a variety of methods described in research literature for measurements of this microbial adsorption and biofilm accumulation (see e.g. Duddridge 1981; Characklis et al. 1982).

Pseudomonas putida is an organism of potential interest in the field of biotechnical processes based on attached biomass. It has a capacity of efficiently forming biofilms under appropriate growth conditions and has a metabolic versatility that may prove important in, e.g., detoxification processes (Molin et al. 1982; Molin and Ternström 1982).

It has been shown that, in a P. putida chemostat, the biofilm build up is strongly influenced by the dilution rate (growth rate). It has also been shown that environmental parameters like pH and temperature affect biofilm build up and cell morphology (Molin et al. 1982; Molin and Nilsson 1983).

The purpose of this investigation was to characterize the biofilm formation capacity of Pseudomonas putida cells produced at different dilution rates (growth rates), in terms of adsorption capacities, flocculation tendencies and hydrophobic properties. This characterization involved a certain development of the methods for estimation of adsorption rates and activities of attached biomass.

MATERIALS AND METHODS

Culture of microorganism. Cultures of Pseudomonas putida ATCC 11172, formerly Pseudomonas fluorescens (Molin and Ternström 1982), were used in all experiments. The freeze-dried cultures were maintained and recovered as previously described. The media and inoculation procedures were also identical with those of the previous study (Molin et al. 1982). The continuous cultures were, with one exception, performed in a 1 l fermentor with a working volume of 0.8 l (Chemoferm AB, Sweden). The flocculation experiment was accomplished in a 4 l fermentor,

working volume 2.5 l (Chemoferm AB, Sweden). The cultures were sparged with 1.0 VVM air and the stirrer speed was 750 rpm. Other equipment used was identical to that previously described (Molin et al. 1982).

The chemostat cultures were run at different dilution rates (D) from 0.1 to 2.1 h^{-1} . At steady state (minimum three times the residence time) OD_{620} was determined and samples were withdrawn and treated in accordance with the procedures described below.

(i) Adsorption of resting cells to glass surfaces and activity of adsorbed cell mass. In order to investigate the adsorption of "resting" cells onto a glass surface and the activity of adsorbed cells, a method based on cell adsorption onto glass slides was developed. The slides (60 x 20 mm) were pretreated with hydrofluoric acid (7.5 M, 5 min), washed and fitted with a rubber o-ring ($\varnothing = 15$ mm). This set was placed in a holder to maintain the o-ring in the right position. This provided a chamber where, within the o-ring, a volume of 1 ml of cell suspension could be applied to the glass slide. The cell suspension withdrawn from the fermentor was harvested by centrifugation, washed twice in 0.9 % NaCl and diluted to OD_{620} -values of 0.1 to 9.0. 1 ml of this cell suspension was exposed to the glass surface for periods of 0.5 to 5 hours. Using a pasteur pipette the cells not attached to the glass were removed and 1 - 5 x 1 ml of 0.9 % NaCl was applied to the o-ring chamber, thereby removing cells loosely attached to the surface. After this the glass slide was supplied with 1 ml of a reaction medium (50 ml 0.9 % NaCl; 1.5 ml of 0.1 M phosphate buffer, pH 6.0; 0.1 g L-asparagine; 0.3 ml growth medium without carbon source (Molin et al. 1982)) and incubated for 90 min at room temperature. Finally, the 1 ml sample was removed and the rise in pH-value of the medium from 6.0 was recorded. An estimate of the activity of the attached cells was made by calculating the $\Delta\text{pH}/\Delta t$ (pH Increase Rate, $\Delta\text{pH}/\text{h}$).

The glass slide was then treated with 2×1 ml of 0.9% NaCl and sonicated (20 + 10 s in an MSE Soniprep 150, ultrasonic disintegrator, MSE, Crawly, England). The 2×1 ml plus the 1 ml original sample were then assayed for optical density (OD_{620}). This last procedure gave an estimate of the total biomass attached to the glass surface.

(ii) Activity of detached biomass. To find a suitable procedure for the activity measurement of biomass detached from a glass surface, samples from the fermentor, (15 ml, washed twice in 0.9% NaCl), were diluted to different cell densities (OD_{620} from 0.05 to 0.2) in 15 ml of reaction medium (see (i)) in an agitated glass vessel. The activity was calculated, after 60 min incubation at room temperature, in terms of $\Delta pH/h$ (pH Increase Rate).

To evaluate the sensitivity of the method, washed samples ($OD_{620} = 0.1$) were heat treated ($70^{\circ}C$, 5 min) and mixed with active cell suspension ($OD_{620}=0.1$) in different proportions. These suspensions were diluted in 15 ml of reaction medium. These final mixtures were then assayed for $\Delta pH/h$ (pH Increase Rate).

(iii) Biofilm experiments. Glass rods ($\emptyset$ 10 mm) were immersed into the fermentation broth to determine biofilm development and the metabolic activity of the attached biomass. After exposure (5 to 44 hours) in the chemostat at $D = 0.8$ (h^{-1}), the biofilm developed was detached according to procedures previously described (Molin et al. 1982). The biomass was washed twice in 0.9% NaCl and diluted to $OD_{620}=0.1$ in 15 ml of reaction medium. The pH Increase Rate was determined after 60 min incubation at room temperature using the procedure described above (ii). At each exposure time a sample from the fermentation broth was similarly

treated and the activity of this sample was in each case set at 100% and the activity of the detached cell mass originating from the bio-film was related to this value.

(iv) Hydrophobicity. A simplified Hydrophobic Interaction Chromatography (HIC) procedure was employed to give a relative hydrophobicity value of organisms grown at different dilution rates in a chemostat. Pasteur pipettes were plugged with glass wool and washed with ethanol. The gel, 2 ml of Octyl Sepharose CL - 4B (Pharmacia AB, Sweden), was diluted 1:1 with 25% ethanol and packed into the pipettes. The columns were equilibrated with 0.05 M Tris-HCl buffer (pH 7.0). Samples withdrawn from the fermentor at different dilution rates were washed twice in Tris-HCl buffer and diluted to a cell density of $OD_{620}=0.5$. 1 ml of this cell suspension was applied to the gel column and eluted with 6 x 2 ml of 0.05 M Tris-HCl buffer (pH = 7.0). The eluate was collected in six fractions and assayed for OD_{620} . The degree of hydrophobicity of the cells was expressed as the ratio of cell mass retained in the column to the mass applied. $(\frac{OD_{620} \text{ appl} - OD_{620} \text{ elut}}{OD_{620} \text{ appl}})$. The HIC tests were at all times run in quadruplicates.

(v) Flocculation. To estimate the flocculation capacities of P. putida cells, samples were taken from the continuous culture run at different dilution rates from 0.1 to 2.1 (h^{-1}). 15 ml samples were transferred into a very weakly agitated glass tube. $FeCl_3$ (0.1 mM) was added dropwise to produce visible flocs. The flocculation capacity was estimated by the required addition of $FeCl_3$ (0 to 0.85 ml). The flocculation is given in arbitrary units as (1 - ml of $FeCl_3$ required for production of visible flocs).

RESULTS

To evaluate the methods for estimation of adsorption of resting cells onto glass surfaces and the metabolic activity of the adsorbed cell mass, experiments were performed, varying attachment time, cell density applied, washing procedure etc. Figure 1a shows the effects of the cell density (OD_{620}) applied to the glass surface on the activity of the adsorbed cells (measured as pH Increase Rate). A rapid increase in activity was observed at cell densities up to $OD_{620}=3.0$. In subsequent experiments a cell density of $OD_{620}=3.0$ was used. Figure 1b depicts the influence of the washing procedure on the adsorbed cell mass and its activity. On the basis of this result 2 washes were used in the following experiments. The effect of exposure time is demonstrated in Figure 1c. The biomass adsorbed reaches a maximum after 3 h of exposure whereas the activity of the cells decreases after extended exposure periods.

To optimize the activity determinations of the biofilms, according to Materials and Methods (iii), experiments were performed at various cell densities and with different fractions of active, viable cell mass. Figure 2a shows that cell densities of OD_{620} from 0.01 to 0.2 give a reasonable response in activity measurements. The following measurements of activity in cell suspensions containing variable amounts of living cells were performed at the cell density of $OD_{620}=0.1$ (Figure 2b). An almost linear relation between the viability of the suspension and activity values was found.

The methods described above were applied to cell suspensions and biofilms emerging from the continuous P. putida cultures. Table 1 demonstrates the adsorption of resting cells onto glass surfaces and the total activity

of the adsorbed cells, grown at different dilution rates. The same pattern was observed when the different cell suspensions were exposed to glass surfaces for periods of 1 to 5 hours. The P. putida cells, grown at different dilution rates in the chemostat culture, were further characterized with respect to two factors of potential importance to bio-film formation; hydrophobicity and flocculation capacity. The hydrophobic activity of the cells, estimated by the Hydrophobic Interaction Chromatography (HIC), is depicted in Figure 3 a and b. There was a very limited retention of cell mass grown at a dilution rate of $0.1 \text{ (h}^{-1}\text{)}$, while the cells cultivated at higher dilution rates exhibited a more pronounced tendency to interact with the n-octyl groups of the gel. In these cases, the cell mass applied to the columns was almost entirely retained after elution with $6 \times 2 \text{ ml}$ of buffer solution. In Figure 3b the relative hydrophobic activity of cells grown at different dilution rates is summarized.

Figure 4 shows the flocculation capacity of the P. putida cells at different dilution rates. Samples were taken from the rather vigorously agitated fermentor and transferred into the very weakly agitated flocculation tube. At dilution rates of 0.7 to $1.3 \text{ (h}^{-1}\text{)}$ a decrease in shearing forces resulted, after a few minutes, in the spontaneous formation of visible flocs. When the dilution rate was further increased there was again a need for the addition of flocculant.

In order to measure the activity of the biofilms produced in the fermentor, glass rods were immersed into the fermentation broth at the dilution rate of $D = 0.8 \text{ (h}^{-1}\text{)}$. The biofilm build up and the activity of the attached biomass, compared with the activity of the suspended cells, is presented in Figure 5. After 44 hours of exposure a rather

dense (approx. 0.5 mm thickness) biofilm was formed on the glass rod. However, the specific activity of this biomass was low compared to that of a not fully developed biofilm.

DISCUSSION

Whether optimizing the enhancement or the elimination of biofilms there are economic advantages accruing from the effective control of bacterial adhesion and subsequent biofilm formation. The development of a bacterial film is the net result of several processes (Bryers and Characklis 1982). Firstly, the adsorption of organic material onto the clean surface. Secondly, the transport of micro-organisms to the surface. The third process is the adhesion of micro-organisms to the surface. This phase is often divided into a more or less reversible adhesion, followed by a permanent irreversible attachment sometimes mediated by polymer bridging. The fourth part is the biofilm production based on cellular reproduction as well as on extracellular polymers. The last contribution to the total process is the biofilm detachment. This can be a random removal of biomass due to oxygen or nutrient limitations deep within the biofilm. It is also well known that many factors are important to the adhesion and bio-film processes, e.g., properties of the substratum, the state of the organisms, physico-chemical properties as cat-ion concentrations, temperature etc. There have been several excellent articles written in this field, (e.g. Fletcher 1980; Wood 1980; Ellwood et al. 1982). However, there is a sparsity of data in the literature concerning properties of the cells important to biofilm formation in controlled continuous cultures. In the present investigation methods were developed for the study of some properties of Pseudomonas putida cells, grown at different dilution rates in a chemostat, important to cell adhesion and biofilm formation.

Several methods for the measurement of microbial adhesion and biofilm accumulation have been presented (e.g. Duddridge 1981; Characklis et al. 1982; Pedersen 1982). These methods are based on staining procedures, microscopy, measurement of biofilm thickness, activity etc. However, we feel that the methods described here, based on pH Increase Rate and optical density, provide a simple and efficient means of estimating the initial adsorption and activity of attached biomass. As seen in Figures 1 and 2 a reasonable sensitivity was demonstrated when cell concentrations applied, washing procedures, exposure times or viability of cell mass were varied.

The initial adsorption of P. putida cells, grown at different dilution rates (Table 1), shows the same pattern as previously described for the subsequent biofilm build-up (Molin et al. 1982). Cells cultivated at three dilution rates were examined and the results indicate that flow rates exceeding the $\mu_{\max}$ of the culture have a pronounced effect on the initial adhesion. The rather fast ensuing biofilm build up could, to some extent, be a consequence of that adhesion.

As shown in Figure 3 the surface characteristics, in terms of cell hydrophobicity, of the P. putida cells change drastically when the dilution rate is increased above the $\mu_{\max}$ of the organism. It is obvious that the cells grown at higher dilution rates all emanate from the biofilm and these cells differ in hydrophobicity compared to the cells grown in suspension. It is already known that hydrophobic interaction is one important factor in the initial adhesion of bacteria to air-water interfaces (Dahlbäck et al. 1981). The reason for the change in hydrophobicity when dilution rate is altered is not known but it has been shown for Serratia marcescens, that the presence or absence

of fimbriae is important (Hermansson et al. 1982). These authors also showed that the content of lipopolysaccharide in Salmonella typhimurium was important to the hydrophobicity. Others have again demonstrated that the loss of oligosaccharide components of the lipopolysaccharides gives a significant increase in affinity for hydrophobic surfaces (Rosenberg et al. 1980).

The development of a microbial film depends not only on the attachment of the micro-organisms to an inert surface but also on the aggregation of the individual cells. As illustrated in Figure 4, the P. putida cells flocculated spontaneously when grown at dilution rates from 0.7 to 1.3 (h^{-1}). These results can also be compared with the biofilm build-up at different dilution rates previously presented (Molin et al. 1982). In that investigation, the biofilm formation was dominant at dilution rates from 0.8 to 1.2 (h^{-1}). It is quite clear that the surface properties, in terms of flocculation capacity, also change drastically when the dilution rates are increased to values above the μ_{max} . It should be noted that temperature, motility, shearing forces and the concentration of particles influence the flocculation rate, (Ash 1979). The collision frequency increases as the square of the concentration of micro-organisms. As shown in Figure 4 the number of suspended organisms is decreasing at higher dilution rates but this does not explain the rather drastic change in required flocculant addition. It could be mentioned that the P. putida cells did not, at any dilution rate, exhibit visible capsular polymer production when standard negative staining was performed. However, this does not exclude the limited production of polymeric material. It has been reported previously that small amounts of a possible specific adhesive polymer were produced by organisms attached to surfaces (Marshall et al. 1971) When considering these properties of the cells, it should be

kept in mind that the possible spontaneous flocculation can create problems when, e.g., total counts or conventional viable counts on agar plates are performed.

The biofilm build-up (Figure 5) was similar to that previously described (Molin et al. 1982; Molin and Nilsson 1983). After 24 h of test surface exposure, a total cell mass of $OD_{620} = 0.35$ was attached to the glass rod. This can be compared with $OD_{620} = 0.2 - 0.5$ in the earlier investigations. The activity assay methods described above were applied to the suspensions of detached biofilm. The specific activity of the biomass detached from the glass rod related to the activity of the suspended cell mass in the fermentor decreased with increasing exposure time and thereby with increasing film thickness. This result confirms previous findings (Molin and Nilsson 1983) where an increase in total attached biomass was positively correlated to a decrease in the number of viable cells measured as cfu/cm^2 of test surface. This is also in accordance with other results showing that a film thickness exceeding $100\ \mu m$ did not increase the activity. It was shown by Bungay et al. (1968), by measuring oxygen profiles in biofilms at different substrate concentrations, that respiration may cease at film depths of 50 to $100\ \mu m$ at the different substrate concentrations of the medium. Kornegay and Andrews (1968) found that the substrate removal rates ceased to increase at film thicknesses exceeding $80\ \mu m$. It should also be stressed that as the film continues to grow, regions of dead cells, autolysis and gas bubbles develop in connection with the support surface. This definitely impairs the attachment and causes the film to fall off as a result of e.g., shearing forces. The residual layer grows out but is, as indicated, often of lower viability.

In conclusion, we feel that the strong biofilm build-up capacity exhibited by P. putida cells growing at dilution rates exceeding the μ_{max} is to

some extent explained by the higher initial adsorption, the increasing hydrophobicity and the stronger flocculation capacity of these cells. It is possible that the hydrophobicity is more important to the initial adhesion and that the flocculation tendency more influences the bio-film build-up.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to Professor Nils Molin and Dr. Göran Molin for valuable discussion and to Ms Birgitta Sörenby and Ms Maria Wilhelmsson for their excellent technical assistance.

This study was supported by the Swedish National Board for Technical Development.

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Table 1. Adsorption of resting P. putida cells onto glass surfaces and activity of adsorbed cell mass. The cells are grown at different dilution rates in a chemostat on L-asparagine.

Dilution rate	Adsorbed cell mass ^a	Activity of adsorbed cell mass
(h ⁻¹)	(OD ₆₂₀)	(ΔpH/h)
0.5	0.38	0.33
0.9	0.90	0.55
1.15	1.05	0.60

^aExposure time of cell suspension to the glass surface: 3 h.

FIGURE LEGENDS

- Figure 1 a - c. Adsorption of resting P. putida cells onto glass surfaces and activity of adsorbed cell mass. a: Effect of different cell densities. b: Effect of washing procedures. c: Effect of exposure time.
- Figure 2 a and b. Activity ($\Delta pH/h$) measured in cell suspensions of P. putida. a: Effect of different cell densities. b: Effect of viability of cell suspension (content of living cells).
- Figure 3 a and b. Relative hydrophobicity of P. putida cells grown in continuous culture on L-asparagine. a: Elution of cell mass (OD_{620}) from HIC columns. Cells grown at dilution rates ($\bullet$) 0.1; ($\circ$) 0.5; ($\blacktriangle$) 0.9 and ($\triangle$) $1.15\ h^{-1}$. b: Relative hydrophobicity (calculated according to Materials and Methods iv).
- Figure 4. Flocculation of P. putida cells grown in continuous culture on L-asparagine. ($\bullet$) suspended cell masses (OD_{620}) in fermentor: ($\circ$) flocculation (Flocculation given according to Materials and Methods v).
- Figure 5. Biofilm build-up of P. putida in continuous culture and specific activity of detached biofilm. (Calculations according to Materials and Methods iii).

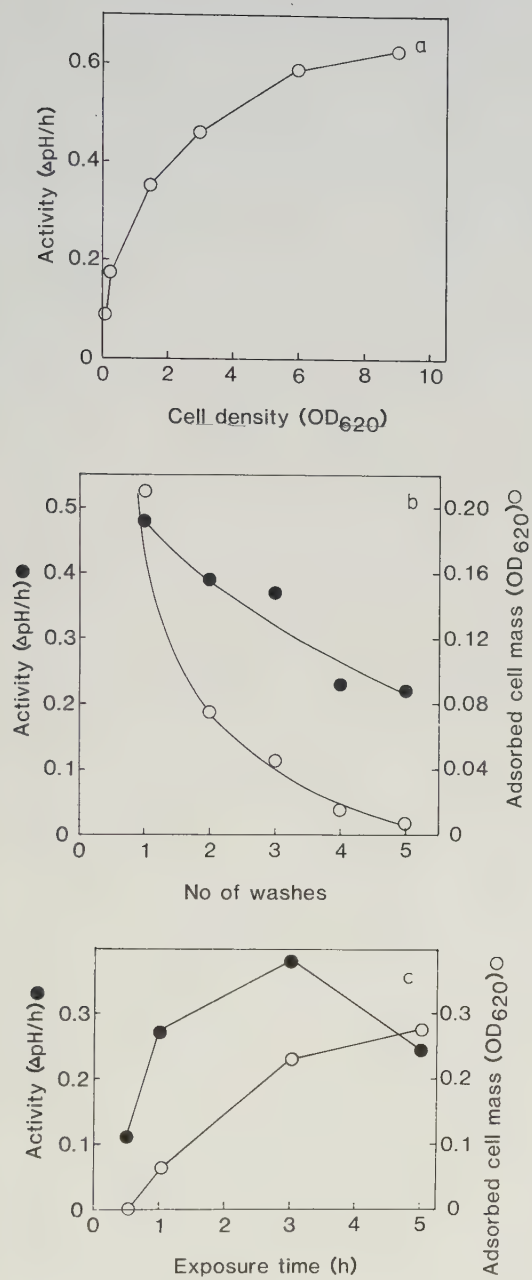


Fig.1

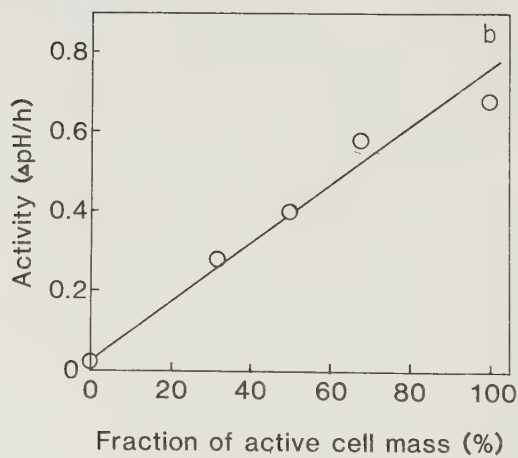
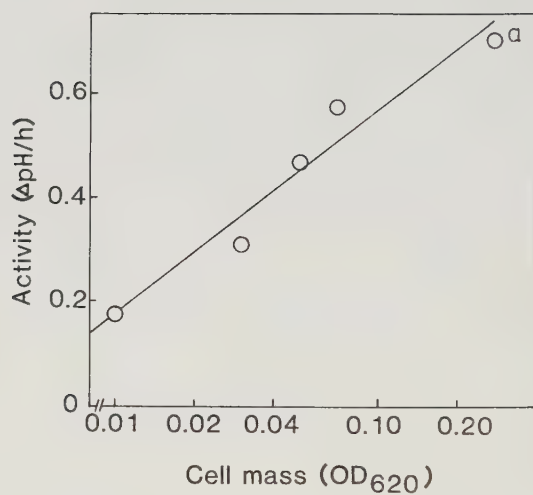


Fig.2

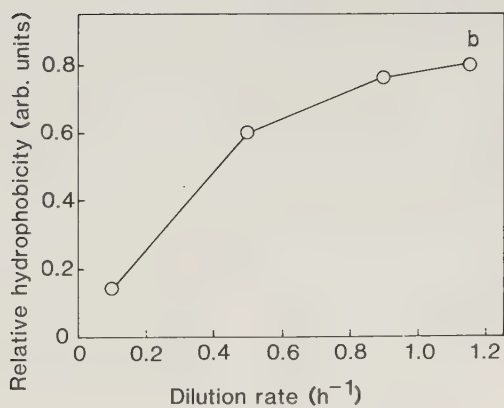
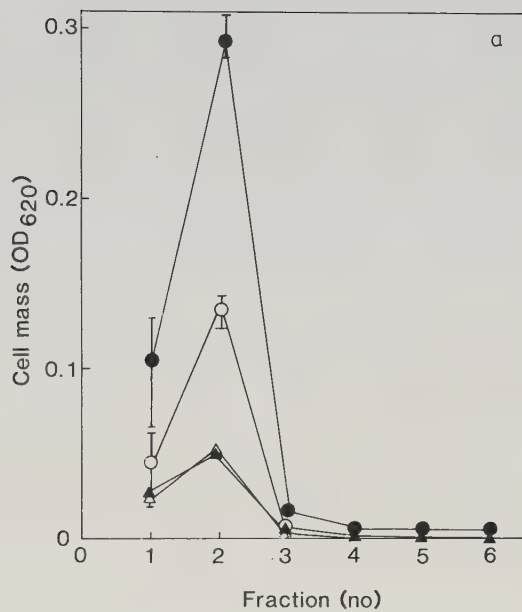


Fig.3

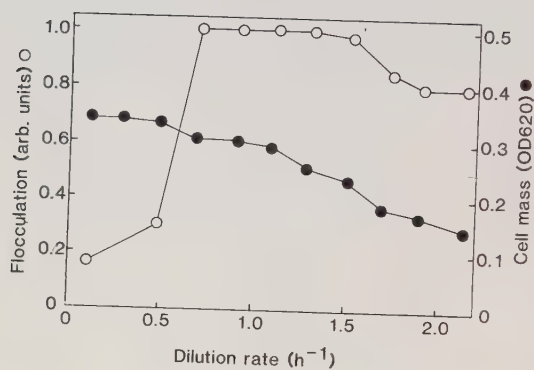


Fig.4

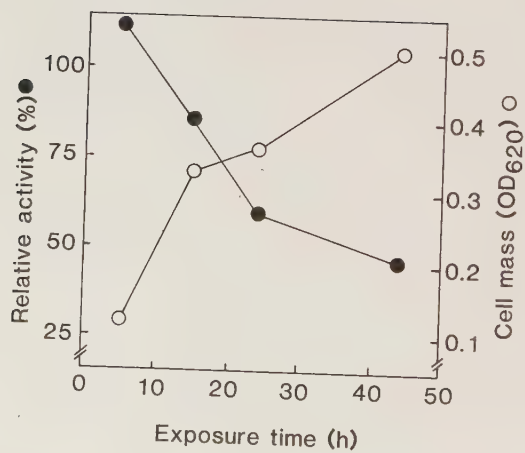


Fig.5

Sand administration as an instrument for biofilm control
of Pseudomonas putida ATCC 11172 in chemostat cultures.

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INTRODUCTION

Since the 1950's, continuous culture has repeatedly been proposed as an exact instrument for microbiological research, and has also been suggested as a favourable principle for industrial fermentation. However, a major obstacle of continuous culture is that the theoretical calculation models have often proved invalid in practice. There are several reasons for this, but one important factor is biofilm formation in the fermentor ^{1, 2, 3}.

Biofilms are common phenomena in continuous cultures and could, for example, be the reason for the unexpectedly high values of $\mu^{\max}$ reported by, for instance, Pirt and Callow⁴ and Cometta et al.⁵ or of the high maintenance coefficient reported by Kuhn⁶.

A fairly common method of delaying biofilm formation on fermentor walls in continuous cultures is to pretreat the fermentor with silicone⁷. However, in the long run this hardly lessens the density of the biofilm, only delaying the biofilm build-up. It has also been suggested that a combination of liquid shear forces and adjustment of environmental parameters should reduce the adhesive forces. However, the problem is that the required conditions may be inappropriate for the fermentation process⁸. A more controlled way of preventing the biofilm build-up is by applying some means of continuous mechanical removal of the attached cells. Scraping devices

can simply be used under non-aseptic conditions, but a mechanical device may be difficult to operate under sterile conditions. A simple way of performing this mechanical removal is by suspending sand in the fermentor.

In general, sand is mostly used as a microcarrier in order to increase the total available surface of attachment in, e.g. fluidized beds, thereby increasing the total biofilm of the system^{9, 10}. The biofilm thickness is here essentially constant as "excess" microbial film is mechanically removed. However, in a vigorously stirred vessel the grinding or shearing effect of the sand particles on all exposed surfaces will increase to a point where all biofilm formation is prohibited.

This work was undertaken with the intention to evaluate the effect of sand administration on the growth characteristics of a Pseudomonas putida ATCC 11172 chemostat. Thus, the characteristics of a culture with and "without" biofilm were compared. The organism was grown at different dilution rates on L-asparagine or phenol as the sole carbon and energy source.

MATERIALS AND METHODS

Microorganism

Pseudomonas putida ATCC 11172, formerly

P. fluorescens¹¹, was used in all experiments.

Stock-cultures were stored freeze-dried in skim-milk at +4°C.

Media and inoculation procedures

L-asparagine or phenol was used as the limiting carbon and energy source in the chemostat cultures. The media and inoculation procedures differed to some extent between the two cultures.

L-ASPARAGINE CULTURES: The organism was recovered, and grown as previously described¹².

PHENOL CULTURES: The growth medium was identical to the asparagine medium except that L-asparagine was replaced with 0.5 g/l phenol, and that the nitrogen content of asparagine was replaced with equal amounts of ammonium sulphate (0.75 g/l).

Cultures on agar slants were transferred to 10 ml of phenol-medium in a baffled flask and then incubated at 25°C for 24 h on a rotary shaker. A secondary culture was prepared by transferring 2.5 ml of the primary culture to 50 ml of phenol medium (25°C for 16 h). In order to ensure the adaptation of the cells to phenol, two more subcultures were performed prior to fermentor inoculation (2.5 ml to 50 ml medium and incubation at 25°C for 24 h and 5 ml to 100 ml at 25°C for 24 h). The inoculum volumes for the 4 l and 1 l fermentor were 100 ml and 50 ml, respectively.

Equipment

The continuous cultures were carried out in two different fermentation vessels; (1) a 4.0 l fermentor (Chemoferm AB, Sweden) with a working volume of 2.5 l, and (2) a 1.0 l fermentor (Chemoferm AB, Sweden) with a working volume of 0.8 l.

The fermentors were ordinary 4 baffled, turbine-agitated vessels (750 rpm). The 1.0 l fermentor was a glass vessel with a top entry stirrer shaft which made it suitable for sand administration. The 4.0 l fermentor was provided with a magnet coupled bottom-entry stirrer shaft (incompatible with sand administration).

The other equipment was identical to that previously described¹². P. putida was grown in carbon source limited chemostats at pH 6.7 and 25°C. The pH was kept constant by titration with 0.6 M NaOH or 0.6 M HCl. The fermentors were, unless otherwise stated, sparged with 1.0 VVM air.

Continuous cultures

The medium flow was started when the batch culture had reached the absorbance (A_{620}) of 0.3 (phenol) or 0.6 (L-asparagine). When the culture reached steady-state (not less than three times the residence time), measurements

were made of; A_{620} , viable count (3 d at 25°C on TGE-agar; Oxoid), dissolved oxygen tension in the culture and concentration of carbon source. Asparagine and aspartate were analyzed enzymatically using the UV-test, (Lebensmittelanalytik, Boehringer, Mannheim, FRG) and phenol was analyzed according to DerYang and Humphrey¹³ using the 4-aminoantipyrine method.

The continuous cultures were run at different dilution rates of 0.1-2.0 h⁻¹.

Biofilm control

In order to minimize biofilm-formation, the 1.0 l fermentor was administered with 50 ml of rough sand particles (mean particle diameter: 0.15 mm). The sand was thoroughly washed in concentrated hydrochloric acid and distilled water to remove impurities. In cultures without sand, the outflow was maintained via an ordinary overflow outlet and a peristaltic pump. To retain the sand in cultures with minimized biofilm build-up, the overflow outlet was replaced by a glass tube ($\varnothing$ 20 mm) immersed 8 cm into the fermentation broth. In this way, the sedimentation rate of the sand exceeded the flow rate in the glass tube. The medium-level of the fermentor was kept constant using a conductivity-type level controller. The eventual loss of sand was controlled by the connection of the discharge to a sedimentation flask which also gave the opportunity of sand recycling.

Biofilm development was elucidated by removing sand particles from the fermentor and examining them in a phase-contrast microscope.

Calculations

The maximum specific growth rate was calculated from the exponential growth phase in batch cultures and by the method of wash-out in the continuous sand-cultures¹⁴.

The saturation constant for the carbon source was calculated according to Pirt¹⁴.

The biomass was, in all calculations, represented by A_{620} -values.

RESULTS AND DISCUSSION

Pseudomonas putida ATCC 11172 was studied in chemostat at different dilution rates and with L-asparagine (Figure 1) or phenol (Figure 2) as the limiting carbon and energy source. The effect of the biofilm on the biomass versus dilution rate curve is demonstrated in the comparison between the biofilm-culture (Figure 1a) and the culture where the biofilm formation was reduced by suspended sand-particles (Figure 1b). In the latter, microscopic examination of sand-particles revealed that no biofilm was present on the surface. "Without" biofilm the biomass-

curve started to decrease in a relatively steep pattern at the dilution rate of the maximum specific growth rate (0.60 h^{-1} ; batch culture), while the biomass curve in the biofilm culture decreased very slowly at dilution rates above 1.0 h^{-1} (1.0 l fermentor) or 0.7 h^{-1} (4.0 l fermentor). Significant amounts of suspended biomass were still present in the biofilm cultures at dilution rates above 1.5 h^{-1} . The general trend was the same in the phenol-culture (Figure 2).

The cultures seen in Figure 1 were performed in two different fermentors where the sand was suspended in the 1.0 l fermentor ("non-biofilm cultures") while the biofilm was allowed to develop in both the 1.0 l and the 4.0 l fermentor. The impact on the steadystate characteristics with a biofilm present in the 1.0 l fermentor is greater than that in the 4.0 l fermentor. This is shown in Figure 1a where the curve of biomass versus dilution rate of the 4.0 l fermentor can be compared with an earlier reported curve from the 1.0 l fermentor¹⁵. The reason for this is that the surface area to volume ratio is about $1 \text{ cm}^2/\text{ml}$ in the 1.0 l fermentor and $0.5 \text{ cm}^2/\text{ml}$ in the 4.0 l fermentor.

The maximum specific growth rate (μ_{max}) of the asparagine-culture was estimated from a "wash-out" experiment ($D = 0.60 \text{ h}^{-1}$ to $D = 0.66 \text{ h}^{-1}$) in the "non-biofilm-culture" and found to be 0.64 h^{-1} , which is close to that measured from a batch culture (0.60 h^{-1} ;

Figure 3a). It is also indicated in Figure 3a that the presence of a dense biofilm makes it impossible to determine $\mu_{\max}$ using the wash-out method. This is in consistence with earlier results^{1, 16}.

Figure 3b shows "wash-out" experiments where the organism was grown on phenol at a dilution rate of 0.3 h^{-1} and the rate was shifted to 0.5 h^{-1} . Almost no wash-out at all occurred in the culture with the dense biofilm, while a $\mu_{\max}$ of about 0.49 could be calculated from the "non-biofilm-culture". $\mu_{\max}$ determined from a batch culture was 0.42 h^{-1} .

A dense biofilm makes it impossible to determine the saturation constant (k_s) for the carbon source using traditional methods. However, in the culture having reduced biofilm the k_s -value for L-asparagine was roughly estimated as being less than 1 mg/l and for phenol about 3.0 mg/l .

This can be compared with k_s -values earlier reported for Pseudomonas aeruginosa, $0.1 \text{ mg aspartate-C/l}$ ¹⁷; for an unidentified species utilizing phenol, 1 mg phenol/l ¹⁸.

The sand administrated culture was continuously run for 7 d and totally during the experimental period the equipment was exposed to the shearing effect of sand for about one month. No negative effects were noticed during this time concerning the pH and oxygen probes or any other "sand-blasted" equipment.

It may be pointed out that the method of biofilm control via sand-shearing can be further developed, concerning the amount of sand, the particle size and stirring speed required. This principle of particle shearing can probably be applied with various other materials, e.g. polystyrene.

Kinetic growth models for continuous biofilm systems have been presented^{3, 19} which may enable a proper description of the system. However, models like these are based on the assumption that certain growth characteristics of the organisms are known.

These vital growth characteristics may be calculated from batch cultures, provided that the characteristics are transferrable from batch to continuous cultures. This is usually taken for granted, but it is possible that the physiological status of an organism is changed enough in continuous culture to influence certain growth characteristics, e.g. the k_s -value. Should this be the case, it is important to be able to determine the growth characteristics of a culture directly in continuous culture, which is very difficult to do in the presence of a biofilm. Anyway, the present method of sand administration also enables the use of the conventional chemostat growth models for organisms with pronounced ability of biofilm formation. Thus, the incorporation of sand into strongly agitated cultures is recommended as an efficient and simple means of controlling the biofilm formation in continuous cultures.

Acknowledgement. The authors wish to thank Ms Lena Stenson-Holst and Ms Maria Wilhelmsson for their excellent technical assistance. This work was financed by the Swedish National Board for Technical Development.

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Figure 1

Pseudomonas putida ATCC 11172 in chemostat with L-asparagine as limiting carbon and energy source.

a 4.0 l fermentor having biofilm: (●) biomass; (○) L-asparagine + aspartate.

1.0 l fermentor having biofilm (earlier study, Molin 1981): dotted lines indicate (▲) biomass and (△) L-asparagine + L-aspartate.

b 1.0 l fermentor having reduced biofilm (sand grinding): (■) biomass and (□) L-asparagine + L-aspartate.

Arrows indicate maximum specific growth rate measured in batch culture.

Figure 2

Pseudomonas putida ATCC 11172 in chemostat with phenol as limiting carbon and energy source: (●) biomass and (○) phenol in a fermentor with biofilm (1.0 l); (■) biomass and (□) phenol in a fermentor (1.0 l) having reduced biofilm (sand).

Arrow indicates maximum specific growth rate measured in batch culture.

Figure 3

"Wash-out" of biomass of Pseudomonas putida ATCC 11172:

- a with L-asparagine as sole carbon source after a shift-up in dilution rate from , (○) 0.6 h^{-1} to 1.8 h^{-1} in a fermentor having biofilm (1.0 l); (□) 0.5 h^{-1} to 0.7 h^{-1} in a 4.0 l fermentor having biofilm; (●) 0.60 h^{-1} to 0.66 h^{-1} in a fermentor (1.0 l) having reduced biofilm (sand).
- b with phenol as sole carbon source, after a shift-up in dilution rate from: (□) 0.30 h^{-1} to 0.50 h^{-1} in a 4.0 l fermentor having biofilm; (○) 0.28 h^{-1} to 0.49 h^{-1} in a 1.0 l fermentor having biofilm. (●) 0.30 h^{-1} to 0.53 h^{-1} in a 1.0 l fermentor having reduced biofilm.

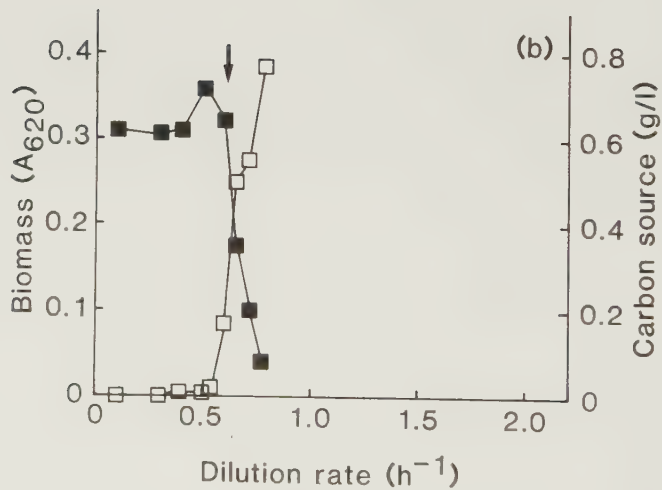
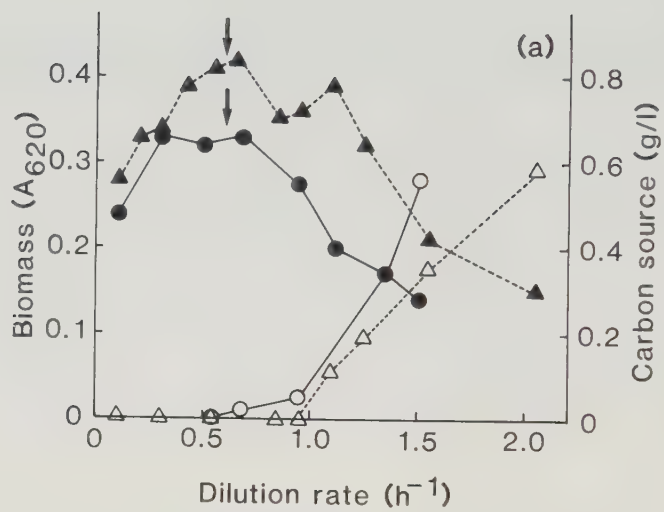


Fig.1

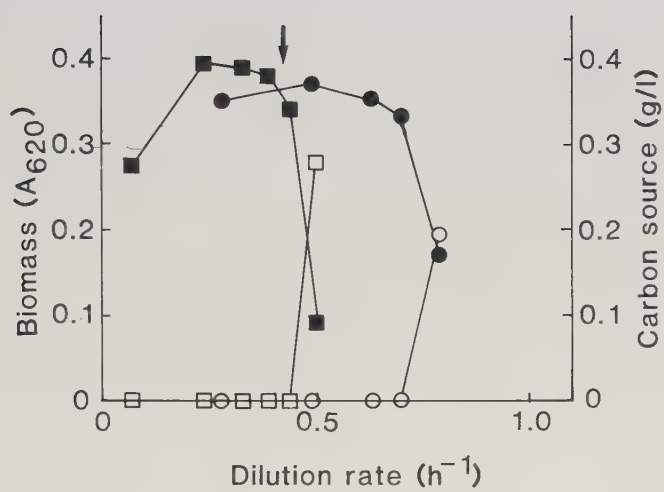


Fig.2

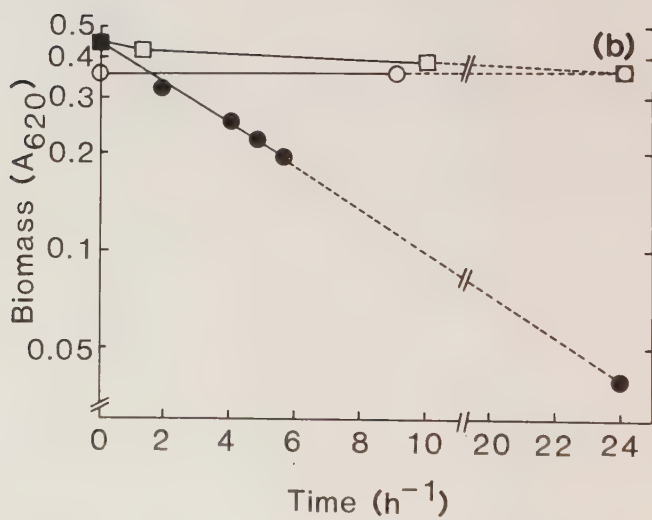
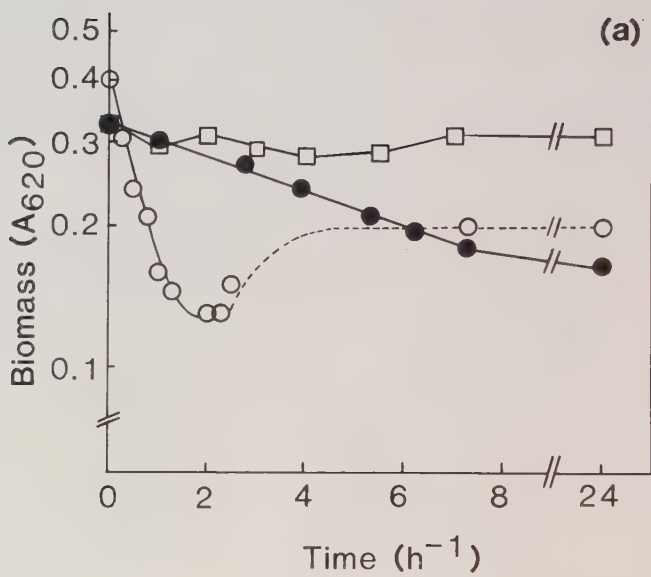


Fig.3

Degradation of phenol by Pseudomonas putida ATCC 11172 in chemostat cultures at different degrees of biofilm activity.

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ABSTRACT

Pseudomonas putida ATCC 11172 was grown in continuous culture with phenol as the only carbon and energy source. The effect of different levels of biofilm activity was evaluated, i.e. the growth characteristics of a culture practically without biofilm were compared with biofilm cultures of different surface-area-to-volume ratios. The variations in ratio were achieved by changing (i) the fermentor volume and (ii) the number of baffles in the fermentor. The biofilms did not significantly influence the maximal cell yield, but they strongly affected the shape of the biomass versus dilution rate curve and, hence, the maximal phenol reduction rate.

In the presence of biofilms the biomass-curve started to decrease at dilution rates far above that of the maximum specific growth rate of the cells ($\mu_{\max}$; 0.4 h^{-1}). At the highest biofilm level (5.5 cm^2 of biofilm surface per ml of reactor volume) the curve started to decrease at a dilution rate of about 1.0 h^{-1} . The maximal phenol reduction rate was increased from $0.45 \text{ g/l}\cdot\text{h}$ ("without" biofilm) to $1.3 \text{ g/l}\cdot\text{h}$ (highest biofilm level), i.e. by a factor of about three. With an increased dilution rate the intermediate product, 2-hydroxymuconic semialdehyde (2-HMA) accumulated in the culture. When the biomass of the effluent started to decrease the concentration of 2-HMA reached a peak-value. 2-HMA has a characteristic yellow colour and the concentration could be determined colorimetrically at 375 nm . It was concluded that the type of continuous biofilm-culture, characterized in the present study, has a potential for the aerobic degradation of aromatic compounds as a stage in the detoxification of industrial wastewaters.

INTRODUCTION

Pseudomonas spp. have a pronounced ability to degrade a wide variety of aromatic compounds, e.g. p-aminoazobenzene (40), azinphos-methyl (17), camphor (8), 3-chlorobenzoate (14), chlorocatechols (24), 4-chloro-2-methylphenoxyacetate (herbicide; 20), 4-chlorophenoxyacetate (18), cresols (7), fensulfothion (pesticide; 37), juglone (32), 4-hydroxyphenylacetate (6), naphthalenesulfonic acids (9), phenol (21), styrene (5), tetralin (34), toluene (19), and 2, 4, 5-trichlorophenoxyacetic acid (herbicide; 10). Most of these compounds may be regarded as hazardous to the environment as they are relatively toxic and resistant to biological degradation. Furthermore, some of these together with other aromatic compounds are discharged into the environment in significant amounts, e.g. effluent from pulp and paper mills (17). With respect to phenol, in particular, this is a common constituent of effluents from polymeric resin production, oil refining and coking plants. Phenol can be toxic to fish at concentrations of 5 mg/l and gives rise to objectionable tastes to drinking water at far lower concentrations (21, 38). There is a general sparsity of data in the literature on the reduction rate of aromatic compounds in continuous systems, even if some reports dealing with the degradation of phenol are to be found (for example, 21, 13). Data such as these are of interest in connection with industrial wastewater treatment.

The productivity of a continuous culture system is increased if the system is based on biofilms instead of freely suspended organisms (39, 25). Furthermore, such a biofilm system is more stable against variations in flow rate (3, 26) and presumably also to sudden concentration increases of, for example, chlorinated aromatic compounds. Biofilms are frequently utilized in aerobic wastewater treatment in reactors of the "trickling filter" and "rotating disc" types (4). However, it has been stated by

Hill and Robinson (21) that wall growth (biofilms) in a chemostat with phenol as sole carbon and energy source decreased the degradation efficiency of the system.

The intention with the present study was to evaluate the effect of different levels of biofilm activity on the growth characteristics of a continuous culture of Pseudomonas putida ATCC 11172 growing on phenol as the sole carbon and energy source.

MATERIALS AND METHODS

Organism. Pure cultures of Pseudomonas putida ATCC 11172, were used in all experiments. The freeze-dried cultures were stored at +4°C. Strain ATCC 11172 is in the American Type Culture Collection (Catalogue of strains I 15th ed. 1982) labelled as P. fluorescens but the organism was reclassified as P. putida biotype I by Molin and Ternström (30).

Media. The freeze-dried culture was recovered by transfer to nutrient broth and nutrient agar (Difco, USA). The organism was after recovery transferred into a phenol medium (0.5 g phenol/l) identical to that previously described (28). This medium was used in all experiments. To adapt the organism to phenol prior to fermentor inoculation, four sub-culture steps were performed in shake flasks (28). The final inoculum volumes for the 1 l and 4 l fermentor were 50 and 100 ml, respectively.

Equipment. The continuous cultures were carried out in a 4 l (2.5 l working volume) or 1 l (0.5-0.8 l working volume) fermentor (Chemoferm AB, Sweden). To vary the surface available for attached growth, different numbers of stainless steel baffles were used. The fermentors are normally equipped with 4 baffles but the number could be changed to 24 (4 l fermentor) or 51 (1 l fermentor). The

approximate surface-area-to-volume ratios for the different combinations are shown in Table 1. The other equipment used was identical to that previously described (29). The dissolved oxygen tension of the fermentation broth was alternatively measured using a galvanic oxygen electrode (23) or a polarographic one (Radiometer, Denmark).

In order to have cultures with minimized biofilm formation, the 1 l fermentor was administrated with sand particles according to procedures previously described (28).

Continuous culture. The fermentor was inoculated from a shake flask culture and the continuous medium flow was started when the optical density of the batch culture was $A_{620} = 0.40$. The fermentation pH was maintained at 6.7 by titration with 0.6 M NaOH and the temperature was held at 25°C. The stirrer speed was kept at 1000 rpm and the aeration was controlled at 1.0 vvm (volume of air/volume of medium/min), unless otherwise stated. The continuous cultures were run at dilution rates from $D = 0.1 \text{ (h}^{-1}\text{)}$ to $D = 1.55 \text{ (h}^{-1}\text{)}$. At steady state (minimum 3 x the residence time) determinations were made of optical density (OD_{620}), dry weight, dissolved oxygen tension, concentration of carbon source and viable count (3 d at 25°C on TGE-agar) of the fermentation broth. The carbon dioxide concentration in the outlet gas was also measured. Phenol concentrations were determined using the 4-aminoantipyrine method according to Der Yang and Humphrey (13).

Determination of 2-hydroxymuconic semi-aldehyde (2-HMA).

When phenol was degraded a characteristic yellow colour developed in the fermentation broth (absorption maximum at 375 nm, Figure 1a). The first ring fission intermediate in the catabolism of phenol is 2-hydroxymuconic semialdehyde, a compound with an absorption maximum at 375 nm (7).

Cells from the continuous culture on phenol were harvested by centrifugation. Cell-free crude extracts were prepared according to Sala-Trepat and Evans (33). After heat treatment (10 min, 55°C), these extracts quantitatively convert catechol to 2-HMA. Different amounts (5.6 to 67.2 µg) of catechol were mixed with 0.1 M phosphate buffer (pH 7.5) to a volume of 2.9 ml, and 0.1 ml of heat-treated extract was added. After incubation (10 min) at room temperature and centrifugation, the absorbance was read at 375 nm. The schematic absorption spectrum of this suspension is given in Figure 1a. The calibration curve for 2-HMA in the fermentor medium is depicted in Figure 1b.

Determination of oxygen transfer rate (OTR). The oxygen transfer capacity of the 1 l fermentor equipped with 4 or 51 baffles was estimated using the sodium sulfite oxidation method (12), at different stirrer speeds and air flow rates at 25°C and normal pressure conditions. The oxygen concentration of the outlet gas was analyzed using a paramagnetic oxygen analyzer (OA 540, Taylor Instrument Analytics Ltd, UK).

Control of biofilm formation. The biofilm formation in the 4 l, 24 baffled fermentor was evaluated at dilution rates above the $\mu_{\max}$ of the organisms (0.4 h^{-1}) by immersing glass rods into the culture. This gave an estimate of the biofilm produced in terms of (a) total amount of attached biomass, measured as A_{620} of a suspension of detached biofilm, and (b) viable cells as colony-forming units (cfu) per cm^2 of glass surface. The sampling device and procedures have previously been described (29).

In order to study the organisms of the biofilm by scanning electron microscopy, glass cover slips (ϕ 0.9 cm) were attached to the glass rods using silicone glue. After sterilization, the glass rods were immersed into the fermentation broth (4 l fermentor, 24 baffles) for different periods of time at different dilution rates. After

exposure, the cover slips were carefully detached and dipped into phosphate buffer in order to remove medium components prior to microscopy procedures. After being rinsed in phosphate buffer (0.1 M, pH = 7.0), the glass cover slips were fixed in glutaraldehyde (3%) for 2 h. The samples were washed and then dehydrated in water/ethanol and freon/ethanol mixtures. Prior to the sputting procedure the specimens were subjected to critical point drying. The samples were finally examined in the scanning electron microscope.

The total biomass attached to the baffles was determined by drying the "24 baffle package" to constant weight at 105°C. This gave an estimate of the film established at the end of the continuous culture.

RESULTS

The effect of different levels of biofilm activity in terms of different surface-area-to-volume ratios in a continuous culture of Pseudomonas putida ATCC 11172 growing on phenol is shown in Figure 2. Thus, Figure 2a shows the growth characteristics in a chemostat where the biofilm is minimized by the shearing effect of sand, while Figures 2b-2d demonstrate the effect of an increased amount of biofilm. If the dilution rate where the biomass-curve has decreased to 0.30 A_{620} -units is taken as a value of comparison, it can be seen in the figures that this value was 0.45 h^{-1} in Figure 2a; 0.55 h^{-1} in Figure 2b; 0.92 h^{-1} in Figure 2c; 1.15 h^{-1} in Figure 2d. Thus, this value which reflects the growth capacity of the culture, was increased by a factor of 2.6 for the two extremes.

The maximum specific growth rate (μ_{max}) of the culture as measured from a batch culture was 0.4 h^{-1} ,

i.e. a value close to the value of comparison obtained in Figure 2a (0.45 h^{-1}).

The saturation constant (K_s) of phenol was in the sand containing culture roughly estimated to be less than 0.003 g/l .

It is also shown in Figure 2 that the oxygen tension of the culture medium decreased with increasing dilution rates. At high surface-area-to-volume ratios the oxygen tension was down to values of about 1% of saturation. At this point, the phenol concentration in the effluent started to increase (Figure 2c and 2d). At these dilution rates the aeration was increased (1.5-2.0 vvm) but no effects were found in the dissolved oxygen tension readings.

The oxygen transfer rate in the 1 l fermentor equipped with different baffle systems is shown in Figure 3 (surface-area-to-volume ratio : 1.0 and 5.5). At low aeration and stirring speed, the oxygen transfer rate was the same for the two reactor configurations. However, if the aeration was increased from 1 vvm to 2 vvm, the oxygen transfer rate in the 5l baffle system was superior, especially at high stirring speeds.

The difference in phenol reduction capacity ($\text{g/l}\cdot\text{h}$) between the conventional chemostat (minimized biofilm formation) and the 5l-baffle system is shown in Figure 4a. The maximum reduction was reached at the dilution rate of 0.45 h^{-1} and 1.4 h^{-1} , respectively. Thus, the maximum reduction capacity was increased by a factor greater than 3 in the multi-baffle-system.

A yellow-coloured intermediate in the phenol catabolism, 2-hydroxymuconic semialdehyde (2-HMA), accumulated in the fermentation broth. The accumulation at different dilution rates is shown in Figure 4b. It is clear that the

maximum 2-HMA concentration (< 5 mg 2-HMA/l) was obtained at a dilution rate giving the maximum reduction rate of phenol.

The appearance of the P. putida cells in established biofilm at the dilution rate of 0.27 h^{-1} is shown in Figure 5.

The established biofilm in the fermentors exhibited a dry weight of about 1 mg/cm^2 and a viable count of about $2 \times 10^8 \text{ cfu/cm}^2$. By immersing glass rods into the fermentation broth, it was shown that the biofilm reached 10^8 cfu/cm^2 within 24 h at dilution rates of $0.8\text{--}1.0 \text{ h}^{-1}$. This value is essentially maintained throughout the experiment, though the total biomass increases also after more than 24 h of exposure.

DISCUSSION

For technical reasons, two fermentors were used in the present study, i.e. one 1.0 l (Figure 2a and 2d) and one 4.0 l fermentor (Figure 2b and 2c). It was shown that the oxygen transfer rate of the 1.0 l fermentor improved with an increased number of baffles (Figure 3). Similar effects of changes in baffle configuration have earlier been shown (15). Nevertheless, the dissolved oxygen tension of the two reactors fell to 1-2% of air-saturation when the biofilm surface was large and the dilution rates were high (Figure 2c and 2d). This could suggest that at least one of the cultures (Figure 2c; 1%) was oxygen limited at higher dilution rates. The growth of Pseudomonas fragi 72 in chemostat was oxygen limited at dissolved oxygen tensions somewhere below 2% (27). However, higher values giving oxygen limitation have also been reported for other Pseudomonas strains but, as indicated by Enfors and Mattiasson (16), such values are difficult

to evaluate as they can be greatly influenced by the experimental procedures. In our study it is important to note that the significant biofilm build-up capacity can affect the dissolved oxygen tensions measured, i.e. bio-film formation on the electrode membrane may influence the apparent values.

Nevertheless, the oxygen transfer rate is a crucial point in an aerobic, continuous biofilm process intended to be run at maximal productivity. This was experienced in the present laboratory study which after all was performed under highly favourable conditions. The problem with oxygen transfer capacity will probably be even more pronounced on an industrial scale. Fluidized bed reactors have been proposed as an industrial means of achieving "biofilm-fermentation" in general (1, 35) but also for phenol degradation in particular (36, 22). However, in view of the difficulties of oxygen transfer, the fluidized bed may be less suitable for a process with a high demand of oxygen. At least, it is very sensitive to the choice of inert carrier (2). In connection to this it should be stressed that the present "multibaffle-system" was demonstrated to actually improve the oxygen transfer rate compared with a conventional fermentor (Figure 3).

Topiwala and Hamer (39) forecast the "tailing" behaviour of the biomass versus dilution rate curve of a chemostat subjected to biofilm formation. The present study empirically demonstrates this effect in the presence of different amounts of biofilm, i.e. at different surface-area-to-volume ratios in the fermentor (Figure 2). Thus, the actual biofilm-activity per unit-area is assumed to be the same in all cultures, except in the culture containing sand. The biofilm was in the latter nearly eliminated. The efficiency of the "sand blasting" in preventing biofilm formation has been discussed previously (28). The major difference between the three other biofilm cultures is the baffle configuration, i.e. the surface

area covered with biofilm and to some degree the oxygen transfer capacity of the reactors.

The present study challenges the report of Hill and Robinson (21), who studied the behaviour of Pseudomonas putida ATCC 17484 in a phenol restricted chemostat. They reported that biofilm formation in the fermentor caused: (i) a decrease in the suspended biomass, and (ii) oscillations in the biomass concentration at higher dilution rates (above that corresponding to $\mu_{\max}$), and (iii) a lower efficiency of the phenol degradation. The present results oppose all these observations, i.e. the biomass was constant irrespectively of biofilm activity, steady-states were obtained at all tested dilution rates and the maximal phenol reduction rate increased with the amount of biofilm present in the system.

Phenol has a potentially inhibitory effect on cell growth, i.e. if the concentration of phenol in the medium is high enough, it will cause substrate inhibition. A dynamic growth model valid for substrate inhibition is $\mu = \mu_{\max} SK_i / (SK_i + K_s K_i + S^2)$ where S is the substrate concentration, K_i is the inhibition constant for phenol and K_s the saturation constant. This model has been demonstrated by Pirt (31) and showed to fit Pseudomonas putida ATCC 17514 growing on phenol (13). Two "steady-states" could according to this model be valid for each dilution rate at the higher range of dilution rates: (i) if the phenol concentration is below a certain critical value (S_{crit}), the growth characteristics will follow the traditional chemostat pattern, (ii) if the phenol concentration is above S_{crit} the "steady state" will be unstable and the biomass will wash-out unless a biofilm is present. The latter may be one explanation to the oscillating biomass in the study of Hill and Robinson (21).

In the present study the K_s -value was 0.003 g/l which could be compared with other values: < 0.001 g/l (P. putida ATCC 17484; 21) and 0.002 g/l (P. putida ATCC 17514; 13). The K_i -values of the two studies were 0.5 g/l and 0.1 g/l, respectively, i.e. the K_s and K_i values differ by a factor of 20-100, and the phenol concentration of the substrate in the present study was of the same order of magnitude as that of the K_i -value. Thus, it could be expected that the growth characteristic of the present study follows a traditional pattern and that no oscillating effect is noted even at steady-states far above the critical dilution rate for wash out predicted by $\mu_{\max}$. Additionally it could be mentioned that the present $\mu_{\max}$ (0.4 h^{-1}) is slightly below that reported by Hill and Robinson (0.5 h^{-1} ; 21) and Der Yang and Humphrey (0.6 h^{-1} ; 13).

The present study set out to indicate the potential use of Pseudomonas spp. in continuous fixed biofilm reactors for the degradation of toxic aromatic compounds in industrial wastewater. It could be argued that the toxicity of phenol is relatively weak and that it easily can be decomposed. However, the present system should be looked upon as a model and even if the degradation of chlorinated aromatic compounds will proceed at a lower rate, the overall behaviour of the system will be the same. Furthermore, the fission of the benzene-ring is one key step in the degradation of all molecules containing this structure.

Pseudomonas putida ATCC 11172 degraded phenol by the "meta-cleavage" pathway (11). This was indicated by the accumulation of 2-hydroxymuconic semialdehyde (2-HMA). 2-HMA produces a yellow colour visually discernible in the fermentation broth and easily recorded by absorbance measurements at 375 nm. Moreover, the accumulation of 2-HMA followed the reduction rate of phenol, i.e. the con-

centration of 2-HMA reached a peak-value at the dilution rate where the phenol concentration in the effluent started to increase (Figure 4). Thus, measurements of the concentration of 2-HMA could provide a simple means of controlling the culture-system. It should also be pointed out that 2-HMA is often reported as an metabolite in the degradation of chlorinated aromatic compounds by Pseudo-monas (24, 32, 5).

Finally, the present results may have some relevance from a strictly ecological point of view. Thus, it was clearly demonstrated that the capacity for the continuous biodegradation by a microbiological system was greatly improved in the presence of a biofilm. A major part of the microbiological activity in nature occurs in interfaces, i.e. within biofilms. However, many ecological model studies in the laboratory are performed in cultures based on freely suspended organisms. Consequently, it should be kept in mind that maximal microbiological conversion rates obtained in "suspended cultures" could significantly differ from those in nature.

ACKNOWLEDGMENTS

We thank Ms Maria Wilhelmsson for her excellent technical assistance, and the Swedish National Board for Technical Development for financial support.

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Table 1. Approximate surface-area-to-volume ratios for the 4 l and 1 l fermentor equipped with different numbers of baffles.

Fermentor volume (l)	Number of baffles	Available surface (cm ²)	Surface area to volume ratio (cm ² /ml)
4 (2.5) ^a	4	1350	0.5
1 (0.8) ^a	4	800	1.0
4 (2.5) ^a	24	3500	1.4
1 (0.5) ^a	51	2750	5.5

^a working volume

FIGURE LEGENDS

- Figure 1: Determination of 2-hydroxymuconic semialdehyde (2-HMA).
- a) Schematic absorbance spectra of fermentor effluent (continuous line) and 2-HMA in buffer solution (broken line)
 - b) Calibration curve for 2-HMA in the fermentor effluent.
- Figure 2: *Pseudomonas putida* ATCC 11172 in continuous culture on phenol as the sole carbon and energy source. Growth characteristics: biomass, (○); phenol concentration, (●); dissolved oxygen tension, (□); in:
- a) the "absence" of biofilm
 - b) the presence of biofilm at a surface-area-to-volume ratio of 0.5 (4 l fermentor, 4 baffles)
 - c) the presence of biofilm at a surface-area-to-volume ratio of 1.4 (4 l fermentor, 24 baffles)
 - d) the presence of biofilm at a surface-area-to-volume ratio of 5.5 (1 l fermentor, 51 baffles)

Figure 3: Oxygen transfer rate in a 1.0 l fermentor equipped with 4 or 5l baffles (surface-area-to-volume ratio: 1.0 and 5.5, respectively): 4 baffles and 1.0 vvm aeration, (○); 4 baffles and 2.0 vvm aeration (●); 5l baffles and 1.0 vvm aeration, (Δ); 5l baffles and 2 vvm aeration (▲).

Figure 4: Pseudomonas putida ATCC 11172 in continuous culture on phenol as the sole carbon and energy source (0.5 g phenol/l in substrate).

a) Phenol reduction capacity in the "absence" of biofilm, (○); the presence of biofilm at a surface-area-to-volume ratio of 5.5, (●).

b) Concentration of 2-hydroxymuconic semialdehyde (2-HMA) in the "absence" of biofilm (□); the presence of biofilm at a surface to volume ratio of 5.5 (■).

Figure 5: Pseudomonas putida ATCC 11172 cells attached to glass surfaces in a chemostat on phenol at a dilution rate of $0.27 \text{ (h}^{-1}\text{)}$. Exposure time of the glass surface to the fermentation broth is (a) 21 h and (b) 64 h.

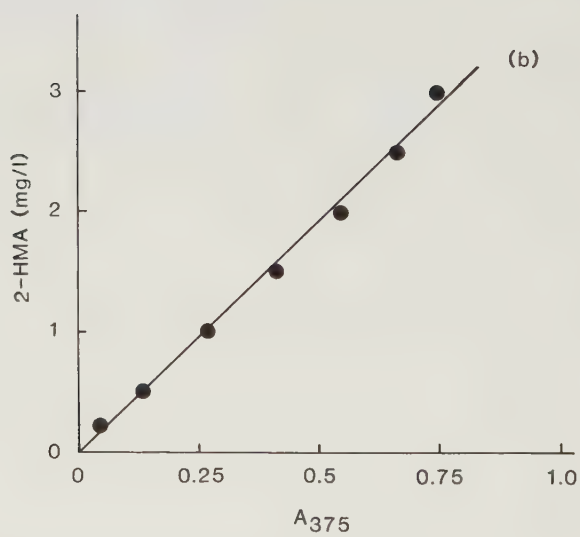
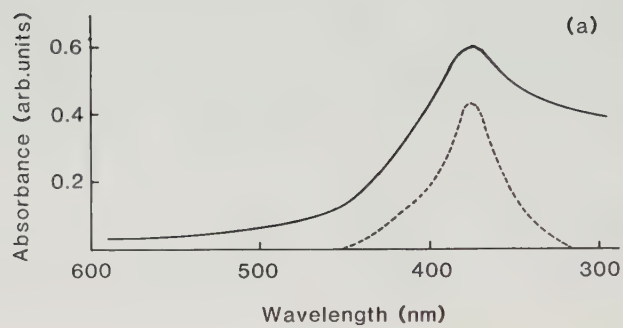


Fig.1

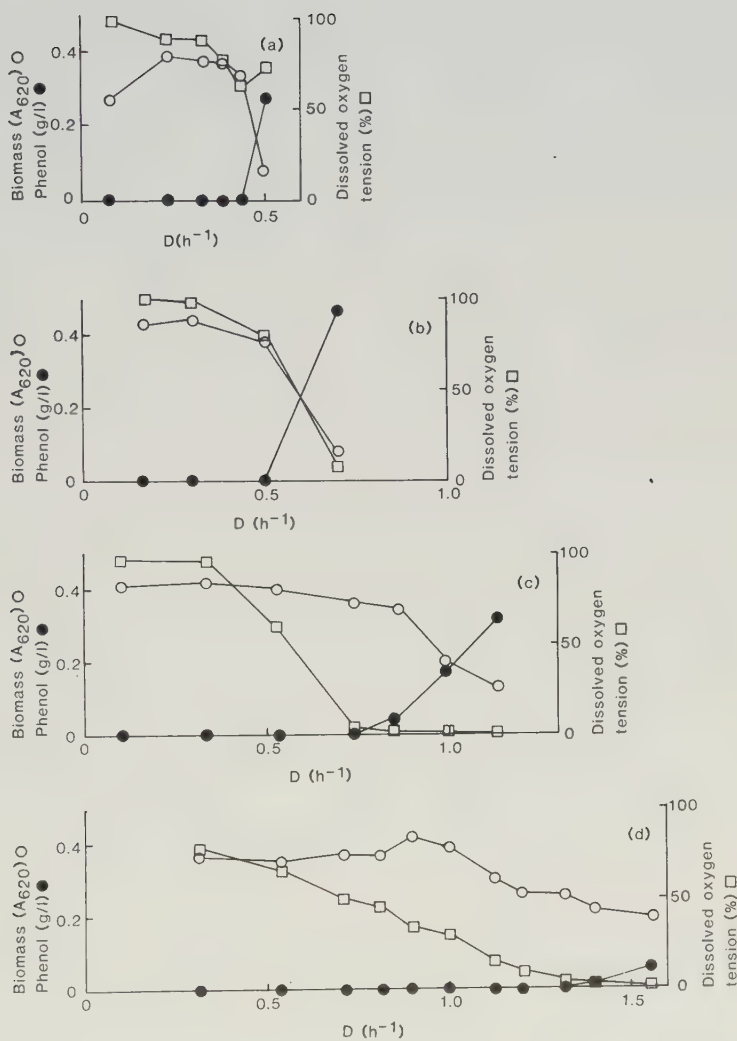


Fig.2

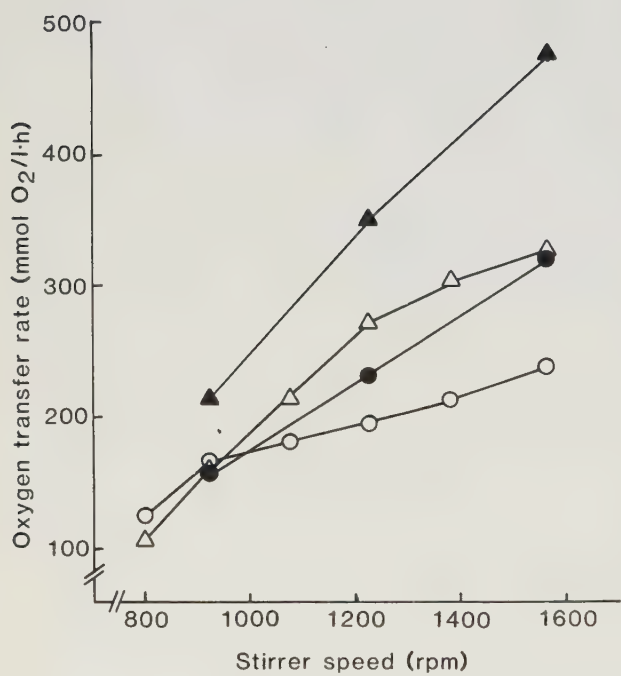


Fig.3

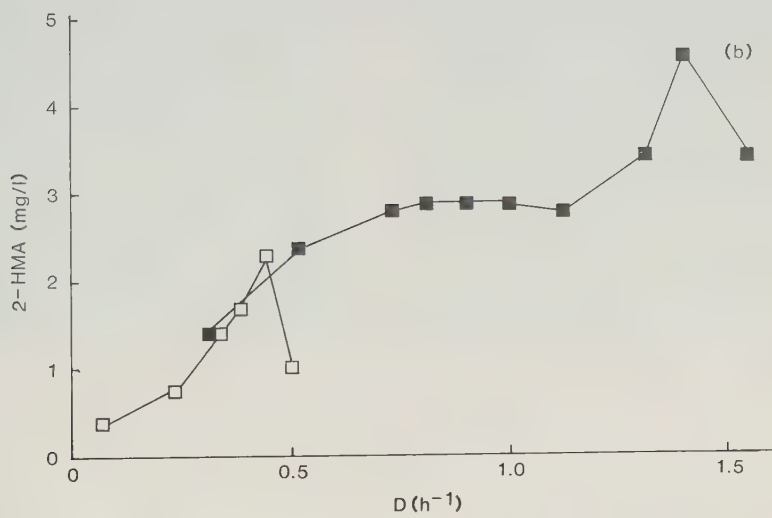
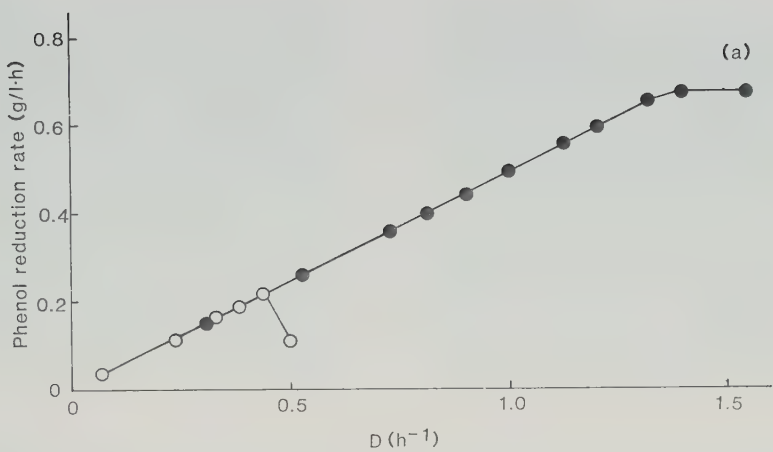


Fig.4

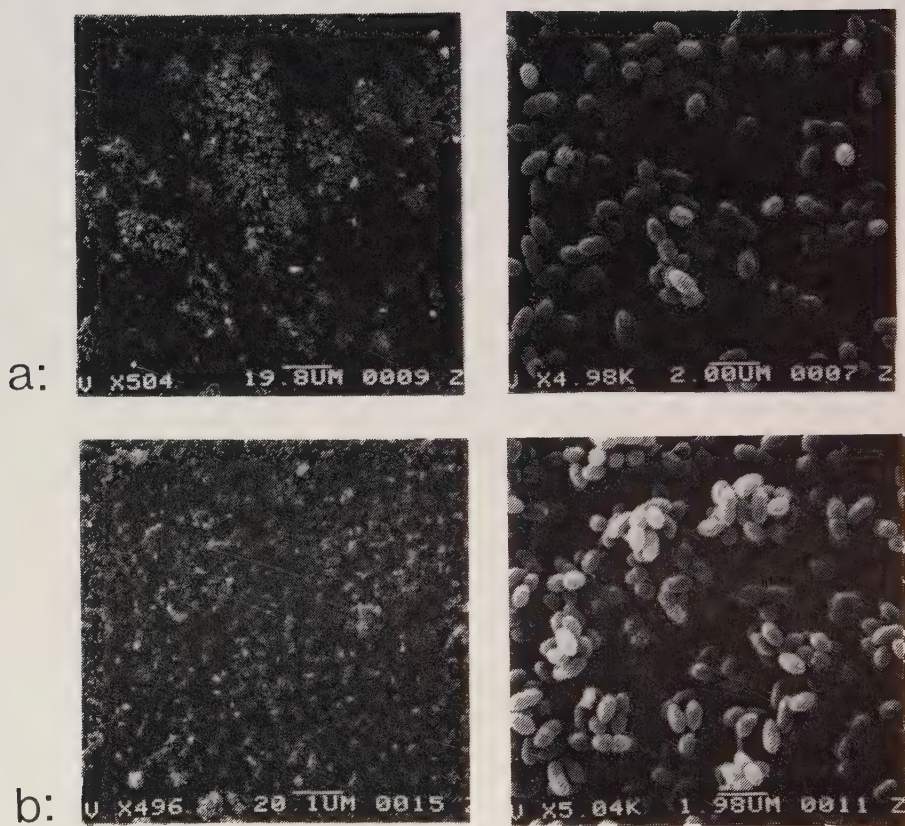


Fig.5

BIOFILM BUILD-UP AND MICROBIAL DENITRIFICATION OF HIGH
NITRATE WATER BY SURFACE ADSORBED PSEUDOMONAS DENITRI-
FICANS CELLS

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Running title: Microbial denitrification by P. denitrificans

ABSTRACT

Pure cultures of Pseudomonas denitrificans ATCC 13867 were studied in aerobic continuous culture on L-asparagine at different dilution rates. The biofilm build-up capacity was investigated at dilution rates above the $\mu_{\max}$ (0.55 h^{-1}) of the organism. The culture exhibited pronounced attached growth at dilution rates above $\mu_{\max}$. The number of cfu attached to glass surfaces increased from $1.5 \times 10^6 \text{ cfu/cm}^2$ to $1.8 \times 10^8 \text{ cfu/cm}^2$ when test surfaces were exposed to the fermentation broth for 0.02 and 68 h at the dilution rate of 0.79 h^{-1} . The number of surface attached cfu stabilized at values about 10^8 cfu/cm^2 .

The biofilm cells, produced under aerobic conditions, showed after a 2 h adaptation period a nitrate reducing capacity similar to that exhibited by cells grown under anaerobic conditions in the presence of nitrate. This biofilm (approx. 1 mg dry wt/cm^2) holding "resting cells" showed a stable denitrifying capacity for at least 1 month. The detrimental intermediate production of nitrite was studied at different flow rates. It was also found that leakage of viable cells from the biofilm was maintained at $10^6 - 10^7 \text{ cfu/ml}$ throughout the experiment. It was shown that lost reduction activity of the respiring P. denitrificans cells can be restored by biofilm regeneration accomplished by means of nutrient addition to the reactor. These results show that the surface adsorbed P. denitrificans cells can be used for nitrate reduction in high nitrate waters.

Keywords: Denitrification, biofilm, Pseudomonas denitrificans, attached growth, biofilm build-up, nitrate reduction, pure water.

INTRODUCTION

The use of surface adsorbed biomass in different biotechnical processes has been intensified in recent years (Messing 1981, Bucke 1983). The development of efficient attached growth processes is intense not least in projects concerning nitrate reduction in water and waste water (Ginocchio 1980; Gauntlett and Craft 1979; Prakasam and Krup 1982). The nitrate content of groundwater in many locations is rising and unacceptable concentrations are quite frequently detected in consumption water (Windle-Taylor 1974). This in turn calls for efficient nitrate reduction methods.

One way of removing nitrate from different waters is to employ microbial denitrification. This anaerobic respiratory process, converting nitrate mainly to nitrogen gas, is performed by many facultative organisms e.g. the genera Micrococcus, Alcaligenes, Pseudomonas and Thiobacillus (Focht and Verstraete 1977). This suggests that, if microbial films are to be used in the denitrification processes, they can advantageously be produced under aerobic conditions and hence a faster biofilm production is achieved.

In general, the adsorption process and the subsequent biofilm build-up depend on many factors, e.g. genetical properties of the organisms, physiological status of the cells, growth conditions etc. (Daniels 1972; Berkeley et al, 1980; Molin et al, 1982; Molin and Nilsson 1983). Other factors of importance are type of substratum used for adsorption, shearing forces etc.

The production and maintenance of the adsorbed biofilm is of particular interest in pure water denitrification, as the water itself does not

contain enough nutrients to sustain the bacterial growth necessary for complete generation and regeneration of the attached biomass. To maintain the denitrification process an exogeneous carbon source has to be added but the amount added is not enough to permit significant bacterial growth.

The purpose of this paper was (1) to study the aerobic build-up of a Pseudomonas denitrificans biofilm in continuous culture and (2) to use the attached biomass for denitrification of high nitrate potable water.

MATERIALS AND METHODS

Micro-organism. Pure cultures of Pseudomonas denitrificans (ATCC 13867) were used in all experiments. It should be noted that P. denitrificans is a genera of uncertain affiliation (Anon., 1982). The organism was stored freeze-dried in skim-milk at +4°C.

Media, inoculation procedures and equipment

The freeze-dried P. denitrificans culture was recovered and grown using procedures previously described for Pseudomonas putida (Molin et al., 1982). The media were also identical to those of the previous study. The experiments were performed in a 1 l fermentor (Chemoferm AB, Sweden) with a working volume of 500 ml. The fermentor was equipped with 51 stainless steel baffles, giving a total surface of 2750 cm² available for attached growth. Other equipment was identical to that previously used (Molin et al, 1982).

Continuous culture. The continuous medium flow was started when the batch culture reached an OD_{620} value of 0.37. The culture was run at different dilution rates from $D=0.15\text{ h}^{-1}$ to $D=1.0\text{ h}^{-1}$. The culture was sparged with 1 VVM air and the stirrer speed was 975 rpm. The pH was kept at 6.7 by titration with 0.6 M HCl and the temperature was 30°C . At steady-state (minimum 3 x residence time), the suspended cell mass was estimated in terms of OD_{620} .

Measurement of biofilm build-up. At the dilution rate $D=0.79\text{ h}^{-1}$ glass rods ($\varnothing$ 10 mm) were exposed to the fermentation broth. The glass rods were pretreated with hydrofluoric acid (7.5 M, 45 min) and immersed into the fermentor for different periods of time. After removal of the glass rods the total attached biomass and the number of viable cells per cm^2 of glass surface were determined according to procedures previously described (Molin et al, 1982). At the end of all experiments the biomass attached to the fermentor glass wall was determined by scraping the surface and suspending the detached biomass in 22 ml of 0.9% NaCl. The number of viable cells was determined by conventional viable count on TGE-agar (25°C , 3d). The total biomass was estimated by drying to constant weight at 105°C . The total biomass, in terms of dry weight, attached to the baffles was also determined by drying the "baffle-package" to constant weight at 105°C .

Denitrification procedures

Determination of Nitrate and Nitrite. Nitrate concentrations were determined using a nitrate electrode (model 93-07, Orion Research, USA). Nitrite concentrations were measured spectrophotometrically according to Standard Methods for the Examination of Water and Waste Water (1975).

Assay of nitrate reduction activity in suspended cell mass. These measurements were performed in order to compare the nitrate reduction capacities of cells grown under different conditions, aerobically or anaerobically, with or without nitrate. Three types of experiments were carried out: (i) with cells removed from the aerobic continuous culture at different dilution rates, (ii) with cells grown anaerobically in 250 ml flasks containing asparagine-medium with 8 g/l KNO_3 and inoculated from agar slants, (iii) like (ii) but inoculum was taken from the continuous culture.

For all experiments, the cells from 60 ml of culture suspension were harvested by centrifugation (12,000 g, 10 min) and washed once in distilled water. The washed biomass was suspended in 15 ml of distilled water supplied with 0.3 ml of 2 M $(\text{NH}_4)_2\text{SO}_4$ -solution and 0.25 g/l K-aspartate. The reaction was initiated by the addition of 50 mg/l nitrate (in form of KNO_3). The progress of the reaction was recorded continuously using the nitrate electrode. The OD_{620} -value of the suspension was measured at the end of the experiment. The specific nitrate reduction rate was determined as the consumption of nitrate per unit of biomass (mg NO_3^- reduced per min per OD_{620}).

Continuous denitrification by surface adsorbed *P. denitrificans* cells.

At the end of the continuous culture, with a *P. denitrificans* biofilm attached to the fermentor surfaces, the fermentation broth was removed. The fermentor was washed once with distilled water and after this a continuous flow of artificial high nitrate potable water (distilled water supplied with carbon source), was started. The sterilized water contained 100 mg NO_3^- /l supplied as KNO_3 and 480 mg K-aspartate/l as carbon source.

The reactor was sparged with nitrogen gas to ensure anaerobic conditions. Stirrer speed was 150 rpm. The dilution rate applied was at most times

$D=0.25\text{ h}^{-1}$ but at certain periods it was varied from $D=0.13\text{ h}^{-1}$ to $D=1.0\text{ h}^{-1}$. At intervals, samples were withdrawn, nitrate and nitrite concentrations and viable count (TGE-agar, 3d, 30°C) were determined. At low reduction activity, growth medium (previously described) was reintroduced to regenerate the biofilm. After regeneration, the flow of high nitrate water was again started.

RESULTS

Biofilm build-up in continuous culture.

To investigate the biofilm build-up capacity and general behaviour of Pseudomonas denitrificans in continuous culture, the organism was grown in an aerated 1 l fermentor at dilution rates from 0.15 to 1.0 h^{-1} . Figure 1 shows the steady-state values of the suspended cell mass. It can be noted that this curve has a somewhat unconventional appearance at dilution rates above the value indicated by the maximum specific growth rate measured in batch culture (0.55 h^{-1}). The biofilm build-up was examined at the steady-state conditions prevailing at $D=0.79\text{ h}^{-1}$. Glass rods were immersed into the fermentation broth and after 0.02, 24 and 68 h exposure they were removed and assayed for the total attached biomass (OD_{620}) and the number of viable cells confined within the biofilm (cfu/cm^2 of glass surface). The 68 h glass rod showed $1.8 \times 10^8\text{ cfu}/\text{cm}^2$ (Table 1). This value can be compared with the number of viable cells attached to the fermentor glass wall at the end of all experiments. After several weeks of operation these glass surfaces exhibited $7.5 \times 10^7\text{ cfu}/\text{cm}^2$. This biofilm was also estimated in terms of total cell mass attached. The glass surfaces held about 0.9 mg of cell mass (dry weight) per cm^2 and the stainless steel baffles approximately 1.2 mg of dry weight per cm^2 .

Influence of the culture conditions on nitrate reduction.

The nitrate reducing capacity of the aerobically grown cells was measured at the different dilution rates (Table 2). In addition, the activity of cells grown under anaerobic conditions in the presence of nitrate was determined. Despite a certain variation, the nitrate reduction capacity of the aerobically grown cells was quite comparable to that of the cells produced anaerobically. However, it should be noted that the cells originating from the aerobic fermentor required a 2 h adaptation period under denitrifying condition before full capacity was reached.

Nitrate reduction by means of surface attached cells.

Having produced a biofilm with nitrate reducing capacity, the growth medium was replaced by artificial high nitrate potable water. It was shown in flask cultures that the P. denitrificans cells did not exhibit any growth when exposed to this medium. This indicated that resting cells were responsible for the nitrate reducing capacity. To be able to study the stability of the reduction capacity the dilution rate was chosen in order to give a trace of nitrite in the effluent. Table 3 reveals a stable reduction capacity for about 25 days. At this time the continuous flow was interrupted. The reduction capacity decreased and the possibility of biofilm regeneration was studied. Growth medium was reintroduced and the dilution rate was increased to values above $\mu_{\max}$ thereby enhancing build-up of active biofilm. After regeneration, the high nitrate water was reintroduced and the denitrification process was run for another 10 days without any substantial decrease in activity.

It is also depicted in Table 3 that the effluent from the fermentor after the initial period held 10^6 to 10^7 cfu/ml. It was however shown in batch experiments that suspensions with this cell density exhibited a negligible reduction capacity, indicating that the attached biofilm was responsible for all reduction capacity.

Finally, Table 4 shows the effect of different flow rates on the nitrate and nitrite concentrations in the effluent. It is also demonstrated that the total denitrifying capacity of the biofilm reactor decreased after regeneration. At higher dilution rates there were substantial concentrations of nitrite in the effluent.

DISCUSSION

The development of a microbial film in a continuous flow reactor can be both advantageous and detrimental. In biotechnical processes based on adsorbed biomass a rapid production of an active biofilm is most important. It is also essential that the mature biofilm can withstand mechanical strain and shearing forces.

It has been shown previously for Pseudomonas putida that one way of promoting biofilm build-up in continuous culture is to apply flow rates exceeding the maximum growth rate of the organism (Molin et al. 1982). Normally the cells should be washed out of the reactor but if the flow rate was slowly increased the organisms soon exhibited a pronounced attachment capacity that prevented the wash out. As demonstrated in Figure 1 the same behaviour was shown by Pseudomonas denitrificans. The shape of the curve of suspended biomass versus dilution rate is probably explained by the increase in biofilm formation capacity. It should be

emphasized that this change in biofilm build-up capacity is not exhibited by all Pseudomonas spp (Molin 1983). As previously stated a great many factors influence the biofilm formation and it is not known what specific factors facilitate this rather drastic change in cell behaviour. It was shown by Nilsson and Dostálek (1984) that for P. putida, properties like hydrophobicity and flocculation capacity changed when dilution rates were increased above the $\mu_{\max}$ of the cells. Hermansson et al. (1982) showed that for Salmonella typhimurium the content of lipopolysaccharides in the cell envelope was important to the cell hydrophobicity. The flocculation capacity is influenced by e.g. production of exopolysaccharides, which in turn is influenced by growth media, oxygen availability, pH, temperature etc (Norberg 1983). This shows, to some extent, that the reasons for the change in biofilm formation capacity are probably very complex.

It is concluded from Table 1 that the biofilm build-up on glass surfaces at higher dilution rates is similar to that shown by Pseudomonas putida in chemostat cultures. The P. denitrificans biofilm contained about 8×10^7 cfu/cm² compared with 1.6×10^8 cfu/cm² for P. putida after 24 h exposure (Molin and Nilsson 1983). The number of viable cells associated with the inert surfaces was stabilized after only 24-48 h. This was confirmed by the tests performed at the end of the experimental run when the glass surfaces held about 7.5×10^7 cfu/cm². This is also in accordance with the previous study by Molin and Nilsson (1983) where the total biomass continued to increase after prolonged exposure of the test surface, while the number of viable cells within the biofilm stabilized at about 10^8 cfu/cm².

The total attached biomass of approximately 1 mg dry weight per cm² would,

assuming a water content of 90% and a biofilm density of 1.0 g/cm^3 , correspond to a biofilm thickness of about $100 \text{ }\mu\text{m}$. This observation could be compared with the "active film thickness" of $100 \text{ }\mu\text{m}$ observed by e.g. Tomlinson and Snaddon (1966). The attached biomass of 1.0 mg/cm^2 could also be compared with the P. putida biofilm produced when phenol is degraded in continuous culture (Molin and Nilsson 1984). That study also revealed a biomass build-up of approximately $1 \text{ mg dry weight per cm}^2$ of baffle surface. It is tempting to suggest that the biofilm build-up to a certain extent adjusts to the prevailing conditions in this reactor, giving an approximate "substrate penetration depth" of $100 \text{ }\mu\text{m}$.

The second purpose of this work was to denitrify high nitrate potable water by means of the attached biofilm. As it is now accepted that denitrification is found only under anaerobic conditions (Bryan 1980) it was important to investigate the nitrate reduction capacity of the aerobically grown cells. Previous work has not fully demonstrated whether the synthesis of nitrate and nitrite reducing enzymes is constitutive or inducible in different organisms. It has also been thoroughly discussed what factors influence the activity of these enzymes (Stouthamer 1976; Bryan 1980). When examining the results in Table 2 it should be noted that samples originating from the continuous culture have the same nitrate reduction capacity as anaerobically grown cells but require adaptation periods from 1 to 2 h. The adaptation period was shorter for samples from the higher dilution rates, where the more dense biofilm perhaps released cells not exposed to high dissolved oxygen tensions. As seen from the last two lines in Table 2, the origin of the inoculum is not too important. Cultures derived from an aerobically grown inoculum showed the same growth and nitrate reduction capacity as did the flask-cultures inoculated from agar slants.

In waste water denitrification the substrate often contains carbon sources and growth factors enough to allow a comparatively rapid biofilm growth and a regeneration of the attached biomass. However, this is not the situation in pure water denitrification, particularly not in this study where the substrate was composed of distilled water, nitrate and K-aspartate. In order to have a rapid biofilm build-up, a defined growth medium was used. As indicated above, a biofilm of approximately 100 μm thickness had established when the growth medium was substituted for high nitrate water. Table 3 shows that the process was run for 25 days without any significant decrease in the activity. For obvious reasons the half-life of the "immobilized" cells could not be established but the stability can be compared with that shown by Nilsson and Ohlson (1982) for columnar denitrification with alginate - immobilized Pseudomonas denitrificans cells, exhibiting a decrease in activity only after 45 days of operation.

The "leakage" of viable cells from the biofilm stabilized after about 3 days at a value of 10^6 - 10^7 cfu/ml. This leakage was at the same level as that (10^6 cfu/ml) shown by alginate preparations in continuous denitrification (Nilsson et al. 1980). It is somewhat surprising that the entrapment procedure leads to approximately the same cell-leakage shown by surface adsorbed biofilms. It is, however, important to realize that the cell leakage is highly dependent on the immobilization procedures, flow rates, shearing forces etc.

It is also clear from Table 3 that, if the reduction activity is lost, it can easily be restored by a continuous flow of growth medium. Activation or regeneration of microbial activity is a procedure previously described (Mattiasson 1983). As in the present study most activity regenerations with

nutrients are due to microbial growth within the immobilized preparations.

When examining the results in Table 4, on the effects of different flow rates, it must be emphasized that the recommended drinking water standard (Sweden) permits 30 mg nitrate/l but only 0.02 mg nitrite/l in water for human consumption. As seen in Table 4 a significant reduction in nitrate concentration could be achieved at high dilution rates. However, at these dilution rates the water contained unacceptable concentrations of nitrite. It was also clear that the regenerated biofilm did not have the same reduction capacity as the original biofilm at higher dilution rates.

To maintain low nitrite concentrations in the effluent, dilution rates above 0.5 h^{-1} could not be applied. The denitrification capacity of this P. denitrificans biofilm reactor (36 mg nitrate-nitrogen reduced/min/g dry weight of biomass) is however comparable to that shown by Gauntlett and Craft (1979) in a fluidized sand bed.

Others have shown that microbial denitrification of pure water by surface attached biomass is a feasible process (Klotter 1969; Gauntlett and Craft 1979), but it is important to remember that factors like formation of by-products, accumulation of nitrite, leakage of microbial cells impair the use of these systems. Accumulation of nitrite was e.g. found by Vossoughi et al. (1982) at high nitrate concentrations when P. aeruginosa denitrified in a packed bed reactor. The denitrification process has to be combined with control systems to monitor nitrate, nitrite and carbon concentrations, filtering devices to reduce the number of microbial cells etc. All this in order to comply with the standards required for potable waters.

In conclusion, the effect of the flow rate on the production of a biofilm ought to be noted when surface attached organisms are to be evaluated for denitrification procedures concerning pure water. Such biofilms can be used for nitrate reduction in pure waters under extended periods of time.

ACKNOWLEDGEMENTS

The author would like to thank Dr Milan Dostálek for valuable discussion and Ms Maria Wilhelmsson for her excellent technical assistance.

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Table 1. Biofilm build-up of P. denitrificans on glass in continuous culture at $D = 0.79 \text{ h}^{-1}$.

Exposure time (h)	Biofilm build-up	
	viable cells (cfu/cm ²)	total biomass (OD ₆₂₀)
0.02	1.5×10^6	0.01
24	8.0×10^7	0.35
68	1.8×10^8	0.49

Table 2. Nitrate reduction capacity of suspended P. denitrificans cells grown under different conditions.

Origin of cells ^a	Spec. nitrate reduction rate (mg nitrate reduced/min/OD ₆₂₀)
Cells grown aerobically in continuous culture at $D=0.43 \text{ h}^{-1}$ (i)	0.033
" " at $D=0.56 \text{ h}^{-1}$	0.064
" " at $D=0.79 \text{ h}^{-1}$	0.030
" " at $D=1.0 \text{ h}^{-1}$	0.043
Cells grown anaerobically in flask culture (ii)	0.042
" " (iii)	0.040

^afor details: See Materials and Methods

Table 3. Continuous denitrification by surface attached P. denitrificans cells^a.

Time (h)	Suspended biomass ^b		$\text{NO}_3^- \text{-N}^b$	$\text{NO}_2^- \text{-N}^b$
	total biomass (OD_{620})	viable count (cfu/ml)	(mg/l)	(mg/l)
0	0.13	n.d. ^c	22.6	0.0
20	0.12	6.7×10^7	< 0.5	0.30
71	0.018	3.1×10^7	< 0.5	0.12
145	< 0.01	9.9×10^6	< 0.5	0.11
189	< 0.01	8.9×10^6	< 0.5	0.11
311	< 0.01	1.8×10^7	< 0.5	0.06
410	< 0.01	9.1×10^6	< 0.5	0.11
600	< 0.01	2.9×10^6	< 0.5	0.20
610	< 0.01	n.d.	22.6	0.0
645 - 865	Regeneration of biofilm			
915	< 0.01	n.d.	< 0.5	0.20
1150	< 0.01	n.d.	< 0.5	0.40

^a reactor volume: 500 ml; available surface: 2750 cm^2 ; dilution rate: 0.25 h^{-1} ; $\text{NO}_3^- \text{-N}$ in substrate: 22.6 mg/l

^b values in effluent

^c n.d.: not determined

Table 4. Effects of different dilution rates on the denitrification rates of surface attached P. denitrificans cells.

Dilution rate ^c (h ⁻¹)	NO ₃ ⁻ -N ^a (mg/l)	NO ₂ ⁻ -N ^a (mg/l)	Denitrification rate (mg NO ₃ ⁻ -N reduced to gaseous products/l/h)
0.13	n.d. ^d (<0.5) ^b	n.d. (0.09)	n.d. (2.9)
0.25	<0.5 (<0.5)	0.11 (0.2)	5.5 (5.5)
0.47	<0.5 (<0.5)	0.21 (2.8)	10.3 (9.1)
0.76	5.9 (2.8)	0.60 (13.7)	12.2 (4.6)
1.0	11.3 (3.2)	0.85 (18.9)	10.5 (0.5)

^avalues in effluent

^bvalues in parentheses: after regeneration of biofilm

^creactor volume: 500 ml: available surface: 2750 cm²:

NO₃⁻-N in substrate: 22.6 mg/l

^dn.d.: not determined

FIGURE LEGEND

Figure 1. Suspended biomass in continuous culture of P. denitrificans on L-asparagine. Arrow indicate $\mu_{\max}$ in batch culture.

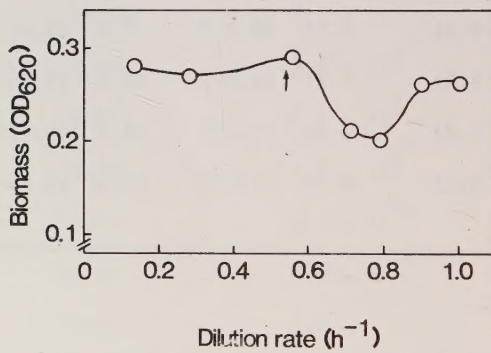


Fig.1

